BIOSYNTHESIS OF

QUINOLIZIDINE ALKALOIDS

A thesis presented in part fulfilment of the

requirements for the Degree of

Doctor of Philosophy

by

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To:- My family

Generations come and go but it makes no difference. The sun rises and sets and hurries around to rise again. The wind blows south and north, here and there, twisting back and forth, getting nowhere. The rivers run into the sea but the sea is never full, and the water returns again to the rivers, and flows again to the sea....

Everything is unutterably weary and tiresome. No matter how much we see, we are never satisfied; no matter how much we hear, we are not content. So I saw that there is nothing better for men than that they should be happy in their work, for that is what they are here for, and no-one can bring them back to life to enjoy what will be in the future, so let them enjoy it now.

Ecclesiastes

Half of the world's population, by virtue of an accident of birth, perform two thirds of the world's work, receive one tenth of its income and own less than one hundredth of its property.

UN Report State of the World's Women 1985

Acknowledgements

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Finally I should like to thank my husband Bill and my mum and dad for their endless support and encouragement throughout this period of my life.

TABLE OF CONTENTS

ABBREVIATIONS

Ac	-	acetyl
br	-	broad
COSY	-	correlation spectroscopy
d	-	doublet
D	-	deuterium (^2H)
DAST	-	diethylaminosulphur trifluoride
DBU	-	1,8-diazabicyclo[5.4.0]undec-7-ene
DIAD	-	di-isopropyl azodicarboxylate
DIBAL	-	di-isobutyl aluminium hydride
DMF	-	N,N-dimethylformamide
DMSO	-	dimethyl sulphoxide
e.e.	-	enantiomeric excess
Et	-	ethyl
i.r.	-	infra red
J	-	coupling constant
m	-	multiplet
Me	-	methyl (CH_3)
m.s.	-	mass spectrometry
n.m.r.	-	nuclear magnetic resonance
Ph	-	phenyl
PPL	-	porcine pancreatic lipase
^iPr	-	isopropyl ($CHMe_2$)
q	-	quartet
s	-	single
t	-	triplet
THF	-	tetrahydrofuran
t.l.c.	-	thin layer chromatography
u.v.	-	ultra violet

SUMMARY

The research presented in this thesis can be divided into four main areas: (a) Biosynthesis of Quinolizidine Alkaloids; (b) Biosynthesis of Ammodendrine and Anabasine; (c) Synthesis of Cadaverine Derivatives and Testing for Biological Activity; and (d) Monoacylation of Diamines Catalysed by Lipases.

(a) Biosynthesis of Quinolizidine Alkaloids

Previous work had revealed that cadaverine is a good precursor of quinolizidine alkaloids. A series of cadaverines, namely (R)- and (S)-[1-^{2}H]cadaverine, [3,3-$^{2}H_2$]cadaverine, [2,2,4,4-$^{2}H_4$]cadaverine, [1-amino-^{15}N, 1-^{13}C]cadaverine and [1,5-$^{13}C_2$, 2,2,4,4-$^{2}H_4$]cadaverine were synthesised by known routes and isolated as their dihydrochloride salts.

The biosynthesis of matrine was studied in Sophora microphylla plants. Samples of (R)- and (S)-[1-^{2}H]cadaverine, (R)- and (S)-[2-^{2}H]cadaverine, [3,3-$^{2}H_2$]cadaverine, [2,2,4,4-$^{2}H_4$]cadaverine and [1-amino-^{15}N, 1-^{13}C]cadaverine were fed with a radioactive tracer to the plants by the wick method. After ten days the plants were macerated and the alkaloids were extracted. Matrine was separated from anagyrine, N-methylcytisine, and cytisine, using preparative t.l.c. plates. High specific incorporations were recorded and labelling patterns were established using ^{2}H and ^{13}C n.m.r. spectroscopy. Information about the enzymic processes involved in matrine biosynthesis was gained and a biosynthetic pathway was proposed.

The biosynthesis of anagyrine was investigated in plants of Anagyris foetida. Specifically labelled (R)- and (S)-[1-^{2}H]cadaverine and (R)- and (S)-[2-^{2}H]cadaverine (prepared by Dr. G.N. Sheldrake) were fed to A. foetida. The alkaloids were isolated and separated using preparative t.l.c. plates to yield anagyrine and N-methylcytisine. Labelling patterns were established by ^{2}H n.m.r. spectroscopy. The stereochemistry of a number of enzymic processes involved in anagyrine biosynthesis was established and labelling patterns previously obtained in N-methylcytisine were confirmed.

3,3-Dimethylcadaverine was synthesised from 2,2-dimethylpropane-1,3-diol by converting the diol into its dimesylate, then displacing the mesylate with cyanide. The resulting dinitrile was reduced using diborane and acidification yielded the final product. Radioactive material was also made using Na^{14}CN. This was fed to A. foetida, S. microphylla and Lupinus luteus plants. N.m.r. spectroscopy and radioactive scintillation counting was used to try to establish if 3,3-dimethylcadaverine is metabolised by the plant. 3-Fluorocadaverine was prepared from diethyl 3-hydroxyglutarate by displacement of the hydroxyl group by fluoride using diethylaminosulphur trifluoride (DAST), followed by reduction using lithium aluminium hydride to the corresponding diol. This diol was then treated with hydrazoic acid, triphenyl phosphine and di-isopropyl azodicarboxylate in tetrahydrofuran, followed by acidic hydrolysis to afford the final product, 3-fluorocadaverine dihydrochloride. This salt was fed alongside a radioactive tracer to L. luteus plants. ^{19}F N.m.r. spectroscopy was used to follow its fate in the plant.

(b) Biosynthesis of Ammodendrine and Anabasine

Samples of (R)- and (S)-[1-^{2}H]cadaverines were used to establish the stereochemistry of the enzymic processes taking place in the biosynthesis of ammodendrine and anabasine.

Ammodendrine biosynthesis was studied in cell-free extracts containing diamine oxidases and in intact leaves and petioles of Lupinus species. GC-MS analysis showed that it is the pro-S hydrogen that is lost and the pro-R hydrogen is retained on oxidation of each cadaverine unit and that similar processes occur in cell-free extracts and intact cells in the formation of ammodendrine.

The biosynthesis of anabasine was investigated in Nicotiana rustica hairy root cultures produced at the A.F.R.C. Food Research Institute at Norwich. ^{2}H N.m.r. spectroscopy revealed that (i) cadaverine is a precursor of the piperidine ring only of anabasine; (ii) it is the pro-S hydrogen that is lost in the conversion of cadaverine into 1-aminopentanal; and (iii) the nicotinic acid attacks the Δ^{1}-piperideine moiety at C-2' from either the re or si face to produce racemic anabasine.

(c) Synthesis of Cadaverine Derivatives and Testing for Biological Activity

A series of cadaverine derivatives was prepared from the corresponding diacid by reduction to the diol followed by transformation of the diol into the diamine using hydrazoic acid, di-isopropyl azodicarboxylate and triphenylphosphine. These diamines were tested by other workers for biological activity against fungi and bacteria. The

derivatives were also made available for testing by others as substrates for the enzyme diamine oxidase.

(d) Monoacylation of Diamines Catalysed by Lipases

Putrescine was successfully monoacylated in ethyl acetate using porcine pancreatic lipase (PPL) as a catalyst. Both the structure of the acyl group and the alkyl group of the solvent ester were found to have an effect on the rate of acylation. The concentration of water and temperature were also found to be significant factors affecting the reaction. Attempts were made to resolve (±)-trans-1,2-diaminocyclohexane, (±)-1,2-diaminopropane and (±)-2-methylcadaverine using the enzymic monoacylation. Some enantioselectivity was observed in the monoacylation of (±)-1,2-diaminopropane when 3-methylpentan-3-ol was used as a solvent with 2 molar equivalents of ethyl acetate.

CHAPTER ONE

INTRODUCTION TO QUINOLIZIDINE ALKALOIDS

At the present time, more than 7000 different alkaloids are known.[1] An enormous variety of structures having many different skeletal types are represented. The term "alkaloid" or "alkali-like" was first coined by W. Meissner, a pharmacist, in 1819.[2] A modern definition was proposed by Winterstein and Trier, who described an alkaloid as a basic, nitrogen-containing compound of either plant or animal origin. "True alkaloids", as they were classed, also fulfilled four additional conditions:

(i) They contained a nitrogen atom as part of a heterocyclic system.

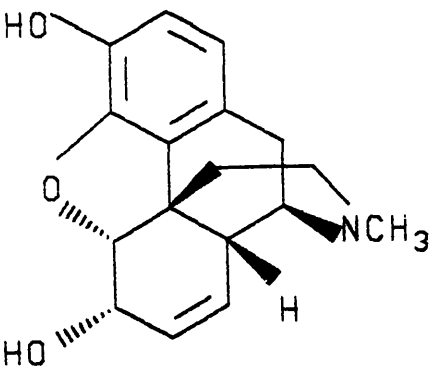
(ii) They had a complex molecular structure.

(iii) They exhibited some kind of pharmacological activity.

(iv) Their occurrence was limited to the plant kingdom.

These "true alkaloids" are restricted to certain families and genera of the plant kingdom - only 9% of over 10,000 plant genera are alkaloid producing. They occur rarely in cryptogamia, gymnosperms and monocotyledons, but are abundant in certain dicotyledons and especially in certain families including Compositae (e.g. groundsel, ragwort), Leguminosae (e.g. broom, gorse, laburnum, lupins) and Papaveraceae (e.g. poppy). Well-known alkaloids include morphine (1) (from the

opium poppy, Papaver somniferum) which was the first pure alkaloid isolated; strychnine (2) (from Strychnos nux-vomica); and quinine (3) (from Cinchona bark).



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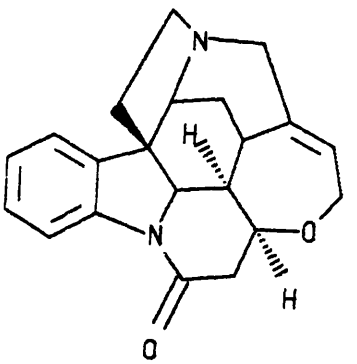

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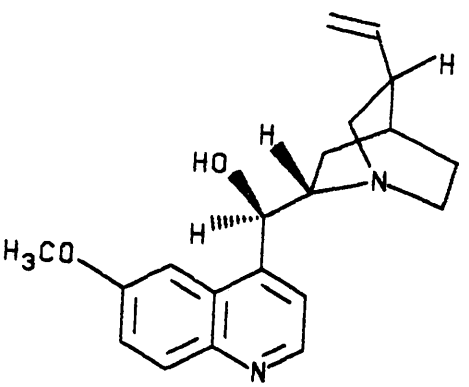

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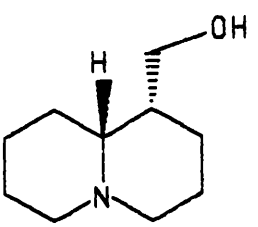
Quinolizidine alkaloids are found in the Leguminosae family.[3] These plants are indigenous to all climates ranging from equatorial rain forests to cold areas and dry deserts.[4] Legumes are not only important as foodstuffs and livestock fodder, but also because they fix nitrogen and help to conserve soil. They are also valuable sources of timber,

oils, resins, gums and dyes.[5] The economic value of legumes is likely to increase rapidly as the human population continues to rise and more marginal agricultural lands are used to produce food. The quinolizidine alkaloids (lupines) form the biggest group of legume alkaloids, and indeed represent about 2% of the total number of alkaloids known from plants. They are important because they are poisonous to humans and to livestock.[6] Furthermore, they show potentially useful pharmacological activities.[7] They can also be used to trace phylogenetic relationships in plant species.[8]

1.1 Structural Types of Quinolizidine Alkaloids

Quinolizidine alkaloids can be divided into six main structural groups:[1,3]

(i) Lupinine (4) and its esters



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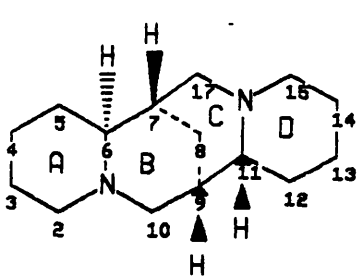
(ii) Tetracyclic quinolizidine alkaloids, such as (+)-sparteine (5) and (−)-lupanine (6). These structures can be modified with a keto group and up to two hydroxyl groups. The hydroxylated lupanines form esters with aliphatic and aromatic acids,



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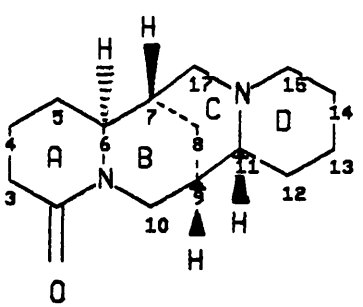

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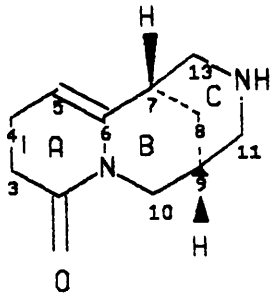
(iii) Pyridone quinolizidine bases, such as (−)-cytisine (7) and anagyrine (8).



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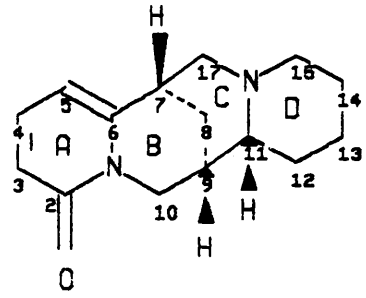

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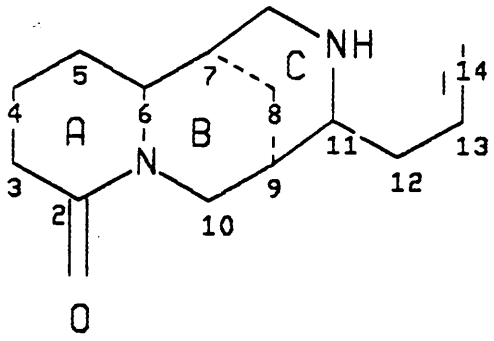
(iv) Tricyclic degradation products of the sparteine/lupanine-type alkaloids, such as angustifoline (9) and tetrahydrorhombifoline (10).



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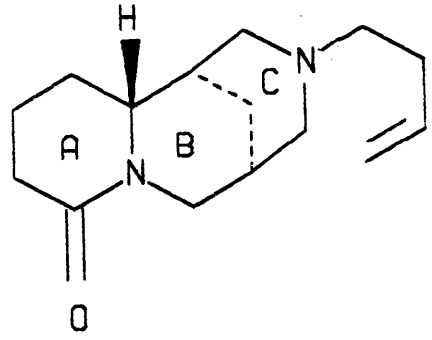

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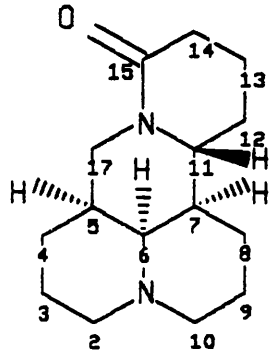
(v) Matrine (11)-type quinolizidine alkaloids.



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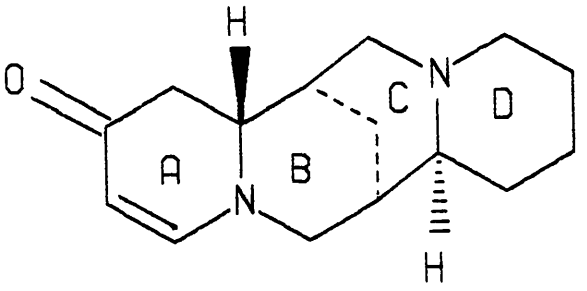
(vi) Multiflorine (12)-type alkaloids with a modified A ring.



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1.2 Methods of Analysis of Quinolizidine Alkaloids

In many cases, thin-layer chromatography (t.l.c.) with detection by Dragendorff's reagent has been used to analyse quinolizidine alkaloids. This method is only of use, however, for simple alkaloid mixtures. Gas-liquid chromatography (GLC) is the preferred method for separation of complex mixtures of alkaloids.[9]

N.m.r. and mass spectrometry are now widely used as well as i.r. spectroscopy which was popular some years ago. N.m.r. spectroscopic techniques used will be discussed in Chapter 2. Mass spectrometry (MS) is often used in conjunction with GLC (GLC-MS), when known structures need to be identified on a small scale.

1.3 Biology and Physiology of Quinolizidine Alkaloids

The biosynthesis of quinolizidine alkaloids (discussed in Chapter 2) occurs only in aerial parts of the plant,[10] although the whole plant accumulates the alkaloids. The highest concentration of alkaloid is found in the seeds - up to 5% dry weight.[11] The accumulation is

tissue-specific, that is, marked concentrations are found in epidermal and subepidermal tissues of stems and leaves.[12,13]

Since quinolizidine alkaloids are found in tissues which do not produce them, a transport system must be present in the plant. Experiments have shown that transportation occurs via the phloem sap in the plants.[14,15]

Quinolizidine alkaloids, like many other secondary metabolites, are not the end products of metabolism, but are in a dynamic state of turnover.[16] This turnover manifests itself in several ways, including the diurnal cycle of the alkaloids (high concentration of alkaloids are present during the day and low amounts are found at night),[17] and the fact that when quinolizidine alkaloids are added to cell suspension cultures they disappear within a week of cultivation.[18]

1.4 Biological Properties of Quinolizidine Alkaloids

Quinolizidine alkaloids are very toxic to range animals, especially sheep.[19] When intoxicated, sheep show symptoms of nervousness, inco-ordination and convulsions. Death may occur as a result of respiratory paralysis.[20] However, since quinolizidine alkaloid intoxication is not cumulative, animals can eat large amounts of legumes containing alkaloids, as long as the lethal dose is not exceeded. The greatest number of livestock deaths due to poisoning happen in the autumn, during the seeding stage. Since the seeds contain the highest level of alkaloid, this fact is not surprising.

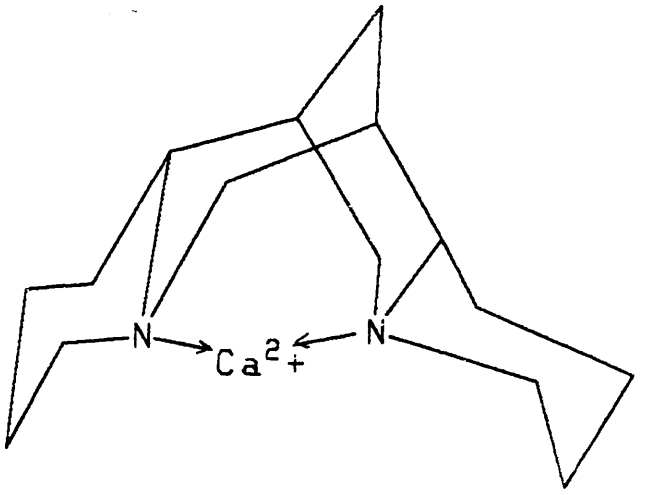
Pyridone alkaloids, such as anagyrine (8) and cytisine (7), have been found to be more toxic than the saturated alkaloids such as

(+)-sparteine (5). Anagyrine (8) causes "crooked calf disease" in cattle, and both anagyrine and cytisine are teratogens in higher animals.[21] Human poisoning with quinolizidine alkaloids is rare, but is reported occasionally.[22]

Below the pharmacological properties of a number of quinolizidine alkaloids are summarised:

Alkaloid	Type of Activity
Sparteine (5)	Antiarrhythmic
	Diuretic
	Uterotonic, Oxytocic
Lupanine (6)	Antiarrhythmic
	Hypotensive
	Hypoglycemic
Anagyrine (8)	Teratogenic (calves)
Cytisine (7)	Teratogenic (chicks, rabbits)
	Respiratory stimulant (nicotine-like activity)
	Hallucinogenic
	Uterotonic, Oxytocic

The pharmacological activity of some quinolizidine alkaloids may be due to their ability to bind to divalent cations such as calcium, e.g. the α-isosparteine complex (13).[23]



(13)

They are able to function as ligands, in a way similar to other diamines.

1.5 Functions of Quinolizidine Alkaloids as Defence Compounds

Quinolizidine alkaloids were formerly regarded purely as metabolic waste products, or forms of nitrogen storage. Nowadays, they are believed to be chemical defence compounds used to ward off predators. Much evidence has been gathered to substantiate this belief:

(i) Alkaloid-free lupin species, when grown together with the alkaloid-rich wild types, were selectively eaten by herbivores.[24]

(ii) The quinolizidine alkaloids lupinine (4), sparteine (5) and lupanine (6) are toxic to, and inhibit growth of, an aphid which normally feeds on alkaloid-free lupins.[25]

(iii) Lupinine is a toxic feeding deterrent of a grasshopper, Melanoplus bivittatus.[26]

Legume species do not have thorns or stinging hairs to defend themselves, making quinolizidine alkaloids a necessary form of defence.

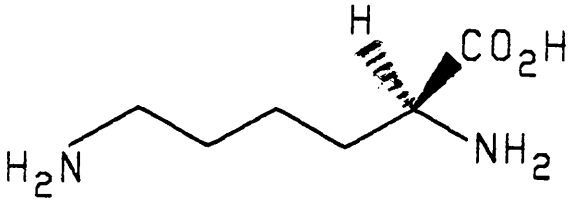
Legumes which do not produce quinolizidine alkaloids often accumulate other toxic substances such as pyrrolizidine alkaloids and lectins, again presumably as defence mechanisms.[27]

Some animals have overcome this defence mechanism, and through evolution, can detoxify quinolizidine alkaloids.[28] They are thus furnished with an ecological advantage over other species. A few aphids have specialised to eat alkaloid-rich lupins - the quinolizidine alkaloids they accumulate act as their defence mechanism. If carnivorous beetles feed on these aphids, they are narcotized within forty eight hours.[29]

The role of quinolizidine alkaloids as a defence mechanism for legumes has, therefore, been established, but this may not be their only role in plants.

1.6 Aims of Project

The way in which quinolizidine alkaloids are formed in plants (biosynthesis) is of fundamental interest. Previously, simple quinolizidine alkaloids such as lupinine (4) and sparteine (5) had received most attention, and their biosyntheses had been studied in depth. They are formed from L-lysine (14) via cadaverine (15), the precursors of six-membered rings containing nitrogen in nature. This area is reviewed in Chapter 2.



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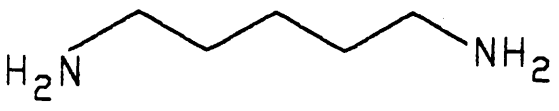

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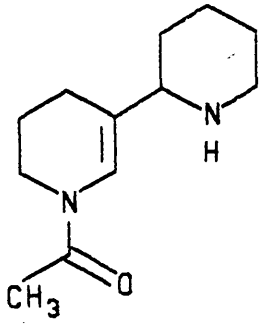
It was considered necessary to widen the scope of these biosynthetic studies. Hence, this research project was directed mainly towards an investigation into the biosynthesis of matrine (11) and anagyrine (8). The results of these investigations are discussed in Chapter 3.

The opportunity arose to study the biosynthesis of two other alkaloids derived from lysine and cadaverine, ammodendrine (16) and anabasine (17). The pyridine ring of anabasine is derived from nicotinic acid (18). It is believed, however, that anabasine can also be formed from two molecules of cadaverine.



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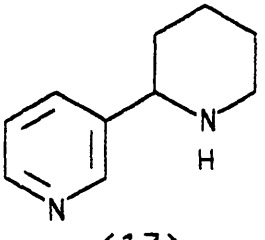

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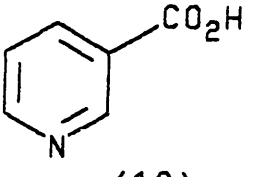

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The results of these investigations are presented in Chapters 4 and 5.

Since quinolizidine alkaloids have a range of biological activities, it is desirable to produce analogues of these alkaloids for biological evaluation. Total synthesis of many of these alkaloids in optically active form is difficult, therefore an alternative strategy was considered. A series of cadaverine derivatives was prepared. It was

intended to use the plants in an attempt to produce analogues of their normal metabolites from the structurally modified biosynthetic precursors. Alternatively, there might be a build-up of analogues of intermediates in the biosynthetic pathway if certain enzyme reactions were inhibited. This approach (described in Chapter 3) suffers from the generation of complex mixtures of analogues alongside the normal metabolites. Tests on some of the cadaverine derivatives, however, showed some interesting biological activities. In addition, these diamines were assessed as substrates for diamine oxidase (DAO) in separate projects supported by S.E.R.C. This work is discussed in Chapter 6.

There is considerable interest in the use of enzymes to resolve racemic mixtures of compounds and to produce optically active material from meso-compounds. Optically active intermediates are extremely valuable in the synthesis of pharmaceuticals in their biologically active form. Investigations were carried out to assess the suitability of lipase as a catalyst for transesterification of one enantiomer of racemic diamines and production of optically active material from meso-diamines. The results of these studies are presented in Chapter 7.

CHAPTER TWO

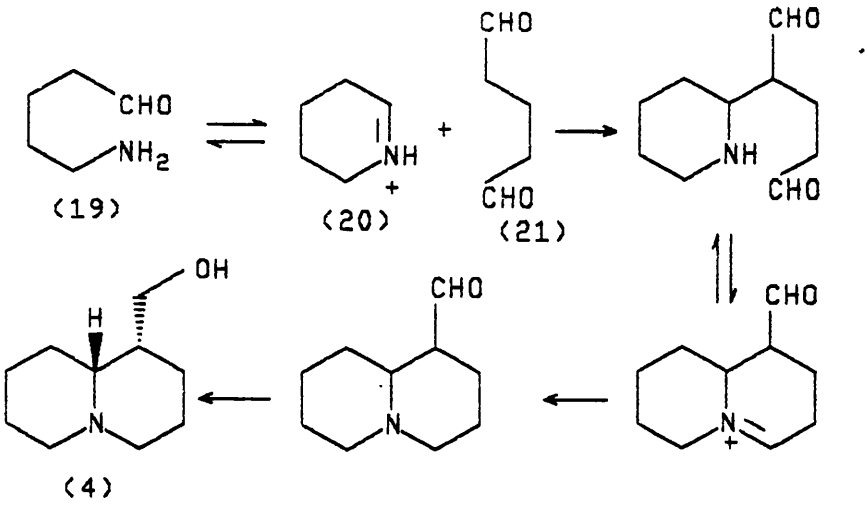
BIOSYNTHESIS OF QUINOLIZIDINE ALKALOIDS

2.1 Introduction

The first rigorous radiotracer studies in natural product biosynthesis were performed more than thirty years ago. These studies served as models for subsequent investigations which have led to the recognition of biosynthetic precursor-product relationships in many classes of compounds. Over the past two decades a number of non-degradative spectral methods have been introduced in conjunction with precursors enriched in stable isotopes which have had a great impact on the methodology for biosynthetic studies. These new tools have assisted in the identification of intermediates which lie on route from precursors to products, and have given insight into the mechanism and stereochemistry of some steps in the transformation of these intermediates. A number of important advances have occurred in the field of research into quinolizidine alkaloids. They will be reviewed in this chapter.

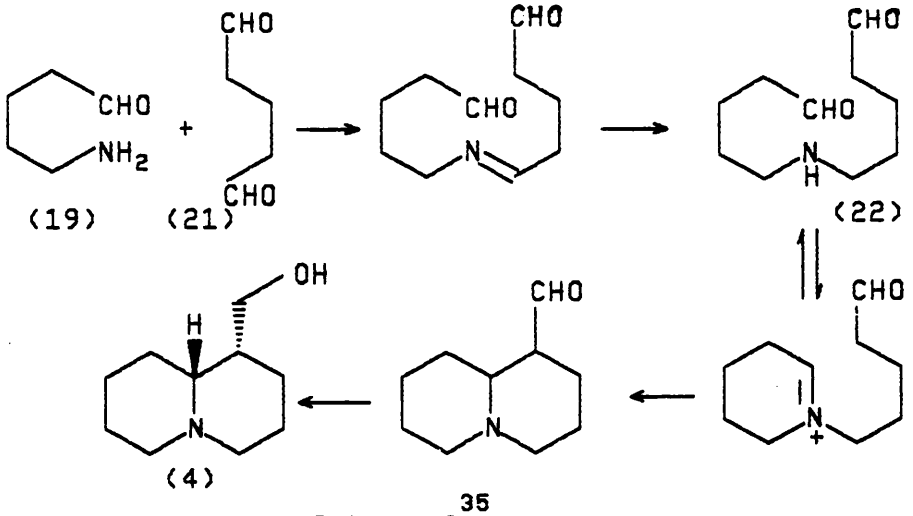
2.2 Biosynthesis of Lupinine

Lupinine (4) is the simplest quinolizidine alkaloid and, as such, has attracted a great deal of attention. Speculations concerning the biosynthesis of lupinine were made more than fifty years ago. In 1931 Schöpf proposed[30,31] that lupinine was formed from two fragments derived from lysine i.e. 5-aminopentanal (19) [$\rightleftharpoons$ Δ'-piperideine (20)] and glutardialdehyde (21) (Scheme 1).[32,33]



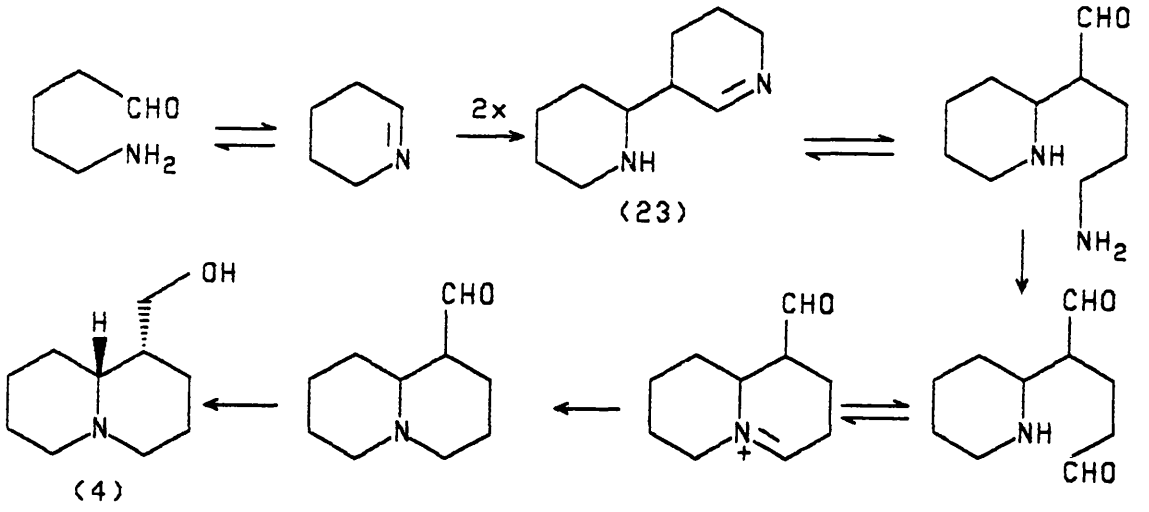
Scheme 1

He later abandoned this theory in favour of another,[32] which involved the intermediate aminodialdehyde (22) between lysine and lupinine. This postulation also found favour with Sir Robert Robinson (Scheme 2).[33]



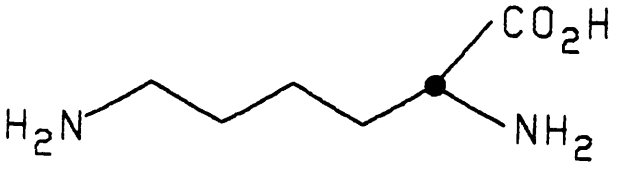
Scheme 2[35]

Another proposal involved tetrahydroanabasine (23),[33] formed from two molecules of Δ¹-piperideine, as an intermediate (Scheme 3).

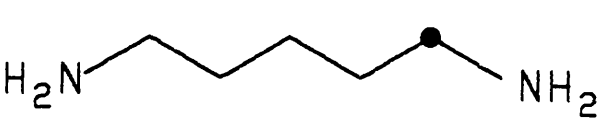


Scheme 3

In order to test these theories, tracer experiments were carried out about thirty years ago. The first experiments involved feeding DL-[2-^{14}C]-lysine (24) and [1-^{14}C]-cadaverine (25) to Lupinus species.[34,36]



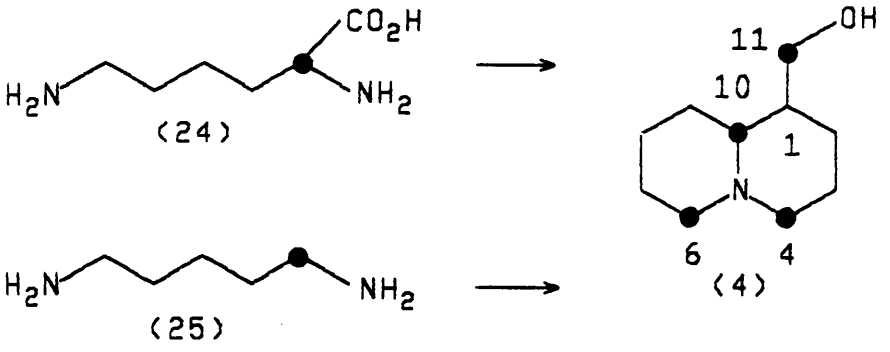
(24)



(25)

● = ^{14}C

Radioactivity from the labelled samples was indeed incorporated into lupinine, and chemical degradation of the labelled lupinine determined some of the sites of radioactive labelling. In both cases, the pattern of labelling was the same (Scheme 4).



Scheme 4

With either intermediate, the hydroxymethylene carbon, C-11, accounted for approximately one quarter of the total activity present in the lupinine molecule. The two methylene carbons adjacent to nitrogen, C-4 and C-6, together accounted for one half of the total activity. It was presumed that each carbon, C-4 and C-6, contained one quarter of the total activity, and the other quarter of total activity was due to the bridge-head carbon, C-10. These results established that lupinine was formed from lysine, and that cadaverine was also a good precursor.

The advent of sophisticated ^{13}C and ^{2}H n.m.r. spectroscopic techniques, opened out the hereto narrow vista of biosynthetic studies. Reliance on the more tedious radiotracer work diminished, to be replaced by techniques employing stable isotopes. Stable isotopes have been used to probe two separate areas of research into the biosynthesis of lupinine:

(i) the structure and symmetry of the intermediates on the biological pathway; and

(ii) the stereochemistry of the processes taking place on the pathway.

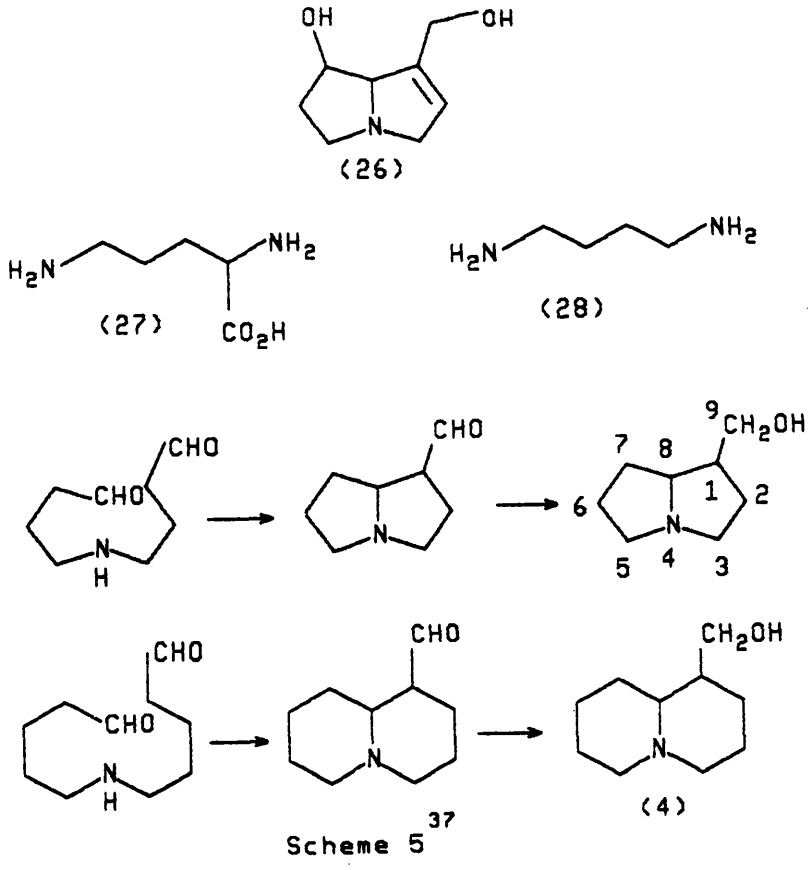
These two topics will be discussed in turn.

(i) Structure and Symmetry of the Intermediates on the Biosynthetic Pathway

The fundamental question in lupinine biosynthesis was whether the pathway was analogous to that of retronecine (26), a pyrrolizidine alkaloid. It was proposed by Sir Robert Robinson that the two compounds arose in the same manner; retronecine from ornithine (27) and putrescine (28), and lupinine (4) from lysine and cadaverine, via dimeric aminodials with C_{2v} symmetry, C_4-N-C_4 and C_5-N-C_5, respectively.[33] These intermediates could undergo an intramolecular Mannich reaction, to produce a pyrrolizidinealdehyde and quinolizidine-aldehyde respectively, which could then be reduced to retronecine (26) and lupinine (4) (Scheme 5).

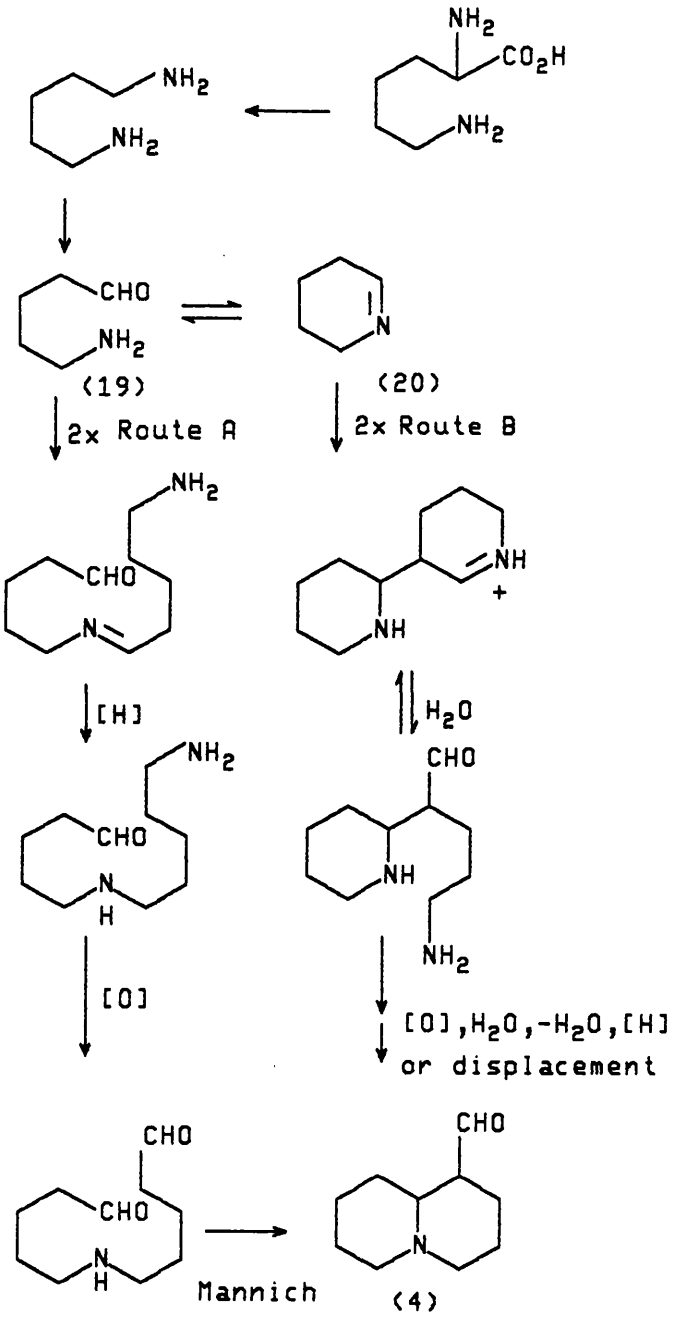
The tracer experiments, described earlier were, in fact, incapable of either confirming or disproving this theory. They were, nonetheless, accepted in support of the postulation.

A further mechanism could give rise to the same radiotracer results, which did not invoke the intermediacy of a "symmetrical dimeric" molecule - this was disregarded at the time. The two plausible routes,



Scheme 5[37]

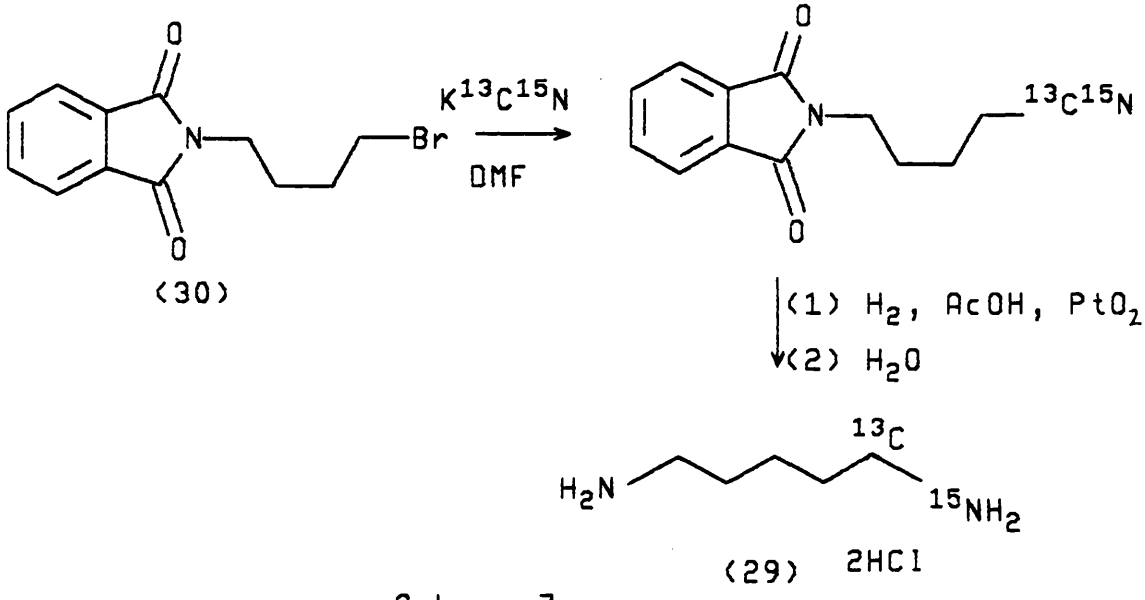
presented in Scheme 6, cannot be distinguished by feeding ^{14}C-labelled substrates. The two routes can be distinguished by the mode of entry of an intact C-N unit from cadaverine. Route A proceeds through a symmetric dimeric molecule, which can yield product in one of two equivalent ways. Route B, on the other hand, has no symmetric molecule as an intermediate, and only one of the three C-N bonds represents an intact C-N bond derived from the precursor. Hence, by labelling the C-N bond in cadaverine the more likely route, A or B, can be determined. The labelling used was ^{13}C-^{15}N - since ^{15}N has spin


NH2
CO2H
NH2
NH2
NH2
CHO
NH2
(19)
(20)
2x Route A
2x Route B
NH2
CHO
N
NH
NH
+
[H]
H2O
NH2
CHO
N
H
CHO
NH
NH2
[O]
[O],H2O,-H2O,[H]
or displacement
CHO
CHO
N
H
CHO
N
Mannich
(4)

Scheme 6[37]

$^1/_2$, any ^{13}C atom with an adjacent ^{15}N appears as a doublet. Due to the natural abundance of ^{13}C, a ^{13}C-^{15}N doublet straddles the ^{13}C natural abundance signal.

The ^{13}C-^{15}N labelling experiment to lupinine was carried out by Golebiewski and Spenser[38] and simultaneously by Rana and Robins.[38] The latter group prepared [1-amino-^{15}N, 1-^{13}C]-cadaverine dihydrochloride (29) by treatment of 1-phthalimido-4-bromobutane (30) with $K^{13}C^{15}N$ followed by catalytic hydrogenation and hydrolysis (Scheme 7)



Scheme 7

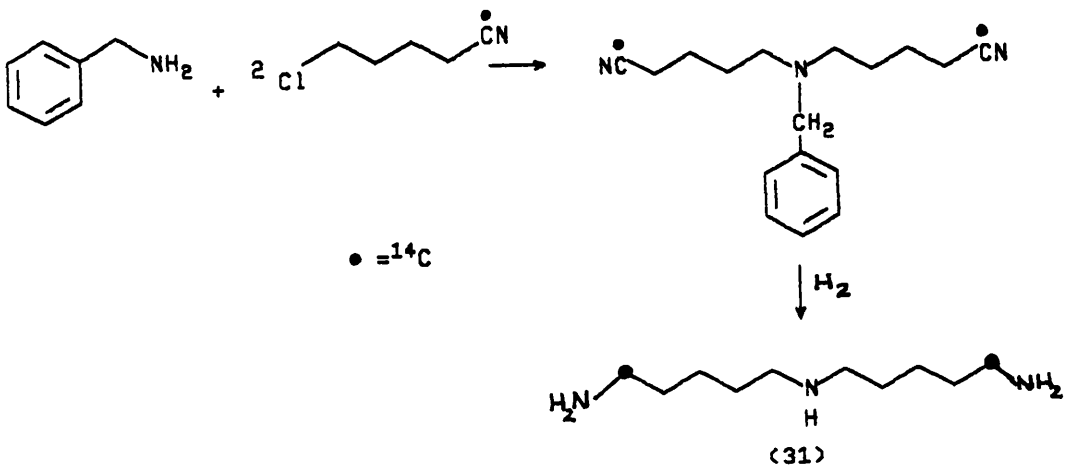
The prepared compound was pulse fed to eight Lupinus luteus plants. Lupinine was isolated and purified by column chromatography, a specific incorporation of 3.9% per C_5 unit being noted. The ^{13}C n.m.r. spectrum showed four enriched carbons at C-4, C-6, C-10 and C-11. Carbon atom C-6 showed an intense ^{13}C-6, ^{15}N doublet, indicating intact incorporation of the ^{13}C-^{15}N unit of the administered cadaverine

into C-6, N of lupinine. Carbon atom C-4 did not show this intense doublet. Similar results were obtained by Golebiewski and Spenser.

Hence this result disproved the theory of the "symmetric dimer" intermediate of C_{2v} symmetry. Lupinine biosynthesis proceeds, therefore, via a dissymmetric dimer intermediate.

This result contrasts with that obtained for retronecine (26) by groups led by Robins and Spenser.[40,41] After feeding [1-^{13}C, 1-^{15}N]-putrescine dihydrochloride to plants of the Senecio genus, retronecine was extracted and purified. Two of the carbons adjacent to nitrogen, C-3 and C-5, appeared as doublets straddling the natural abundance singlets, with identical signal areas. In this case, the biosynthetic pathway does indeed include a symmetric dimeric intermediate with C_{2v} symmetry.

To verify this result Rana and Robins prepared N-(5-aminopentyl)-1,5-diaminopentane (31) labelled with ^{14}C in the terminal carbons (Scheme 8).

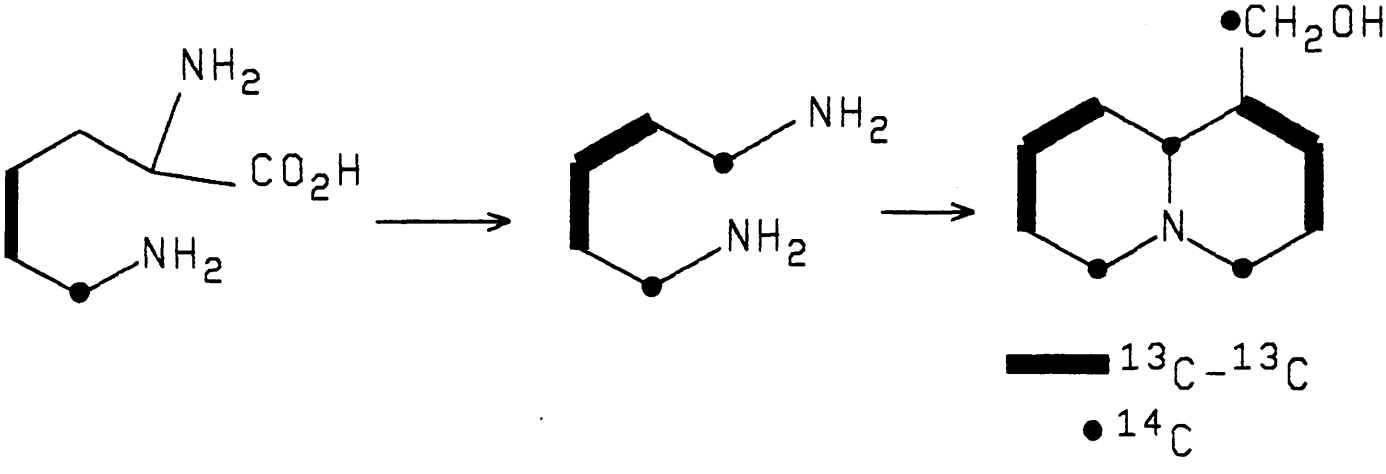


(31)

Scheme 8[43]

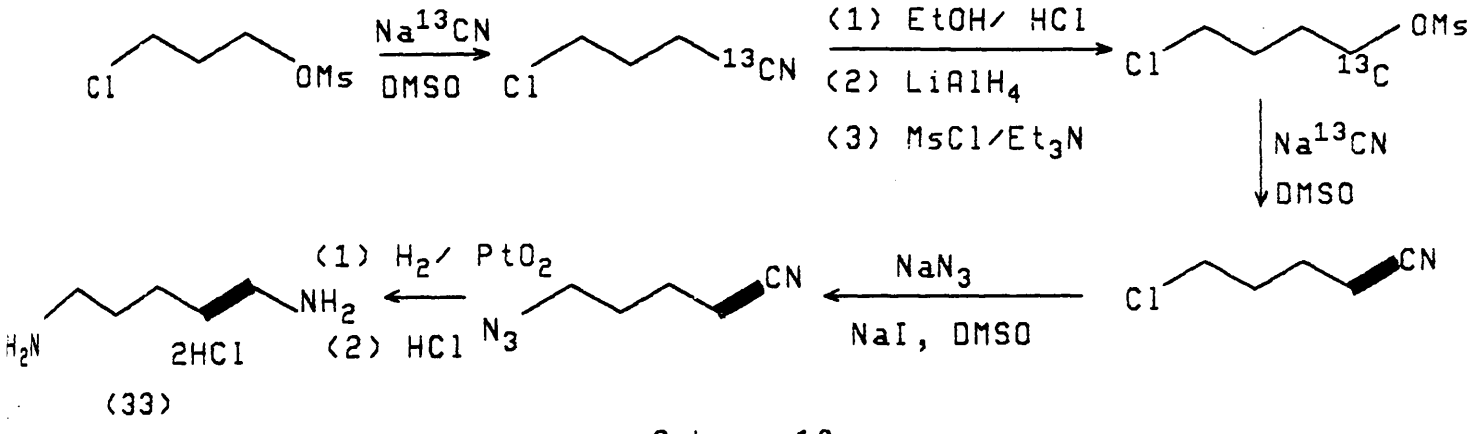
The triamine was pulse fed to nine L. luteus plants over 10 days. After a further 10 days, the plants were harvested and the lupinine was extracted. The lupinine was recrystallised to a constant specific ^{14}C incorporation of 0.04%, suggesting that the triamine is not on the biosynthetic pathway.

Rana and Robins have also performed experiments employing ^{13}C-^{13}C coupling in ^{13}C n.m.r. spectra. DL-[4,5-$^{13}C_2$, 6-^{14}C]-lysine (32) used in biosynthetic studies on the Lythraceae alkaloids[44] and anabasine,[45] was fed to L. luteus plants.[46] The ^{13}C-{^{1}H} n.m.r. spectrum of the isolated lupinine revealed satellites around the six high-field signals due to ^{13}C-^{13}C couplings. The integration of the peaks showed that four carbons were labelled to the same extent, while the other two had approximately double the ^{13}C incorporation. This result is consistent with decarboxylation of the [4,5-$^{13}C_2$, 6-^{14}C]-lysine to produce cadaverine, which was then labelled [2,3-$^{13}C_2$] and [3,4-$^{13}C_2$]. Different combinations give the overall labelling pattern, as shown in Scheme 9.



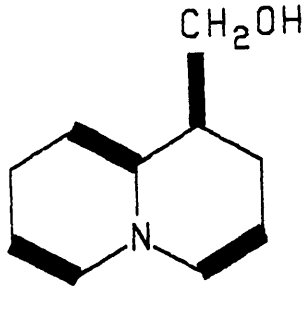
Scheme 9[46]

[1,2-$^{13}C_2$]-Cadaverine dihydrochloride (33) was prepared by Robins and Sheldrake by the method shown in Scheme 10.[47]



Scheme 10

Cadaverine was chosen as the precursor because incorporations are generally much higher than with lysine. The substrate was fed to L. luteus plants and after harvesting, the lupinine was extracted and purified in the normal fashion. The ^{13}C-{^1H} n.m.r. spectrum showed four pairs of doublets and no ^{13}C label at C-2 or C-8, establishing the labelling pattern shown in (34).



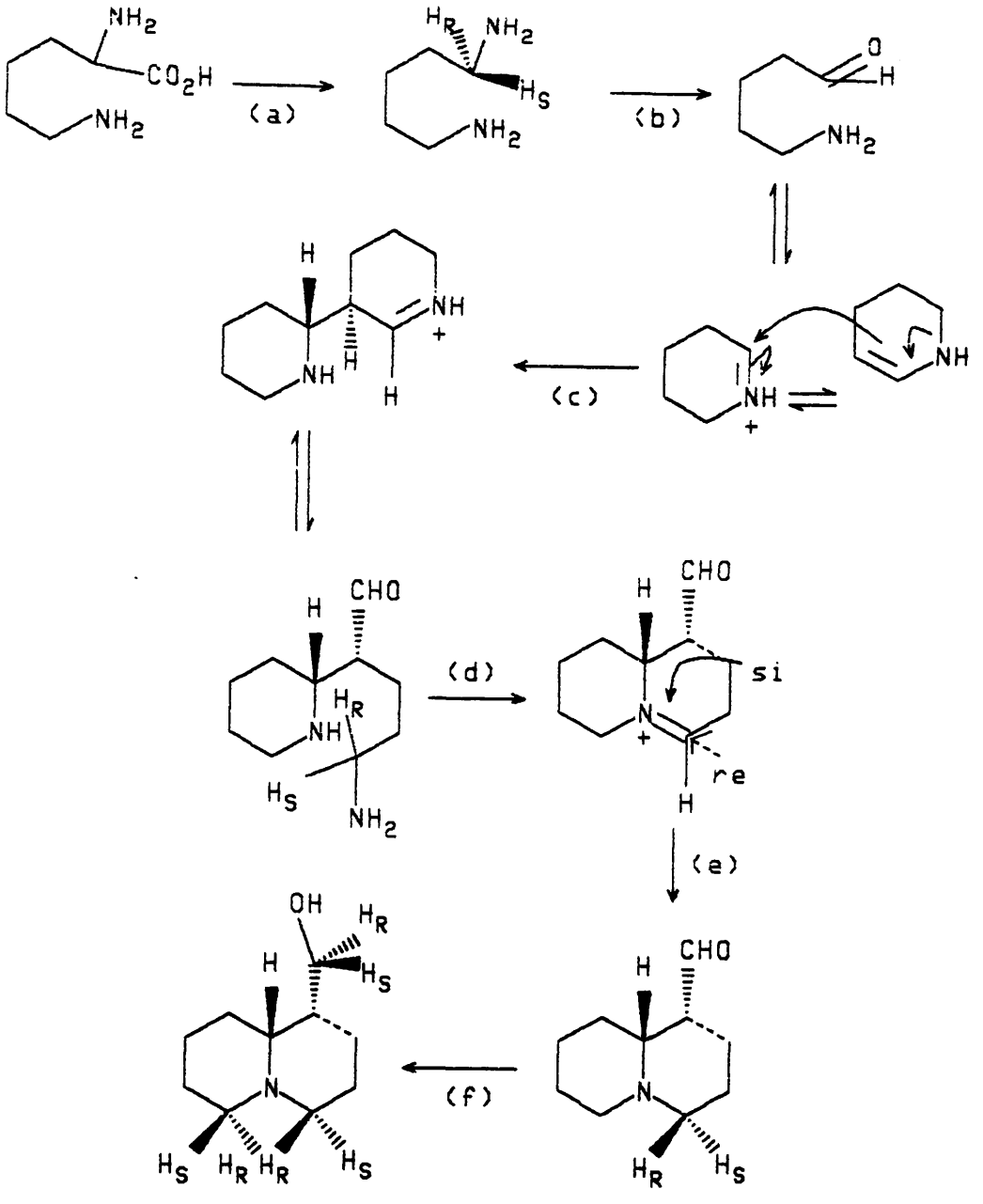
(34)

Again this result confirmed that lupinine is formed from two molecules of cadaverine.

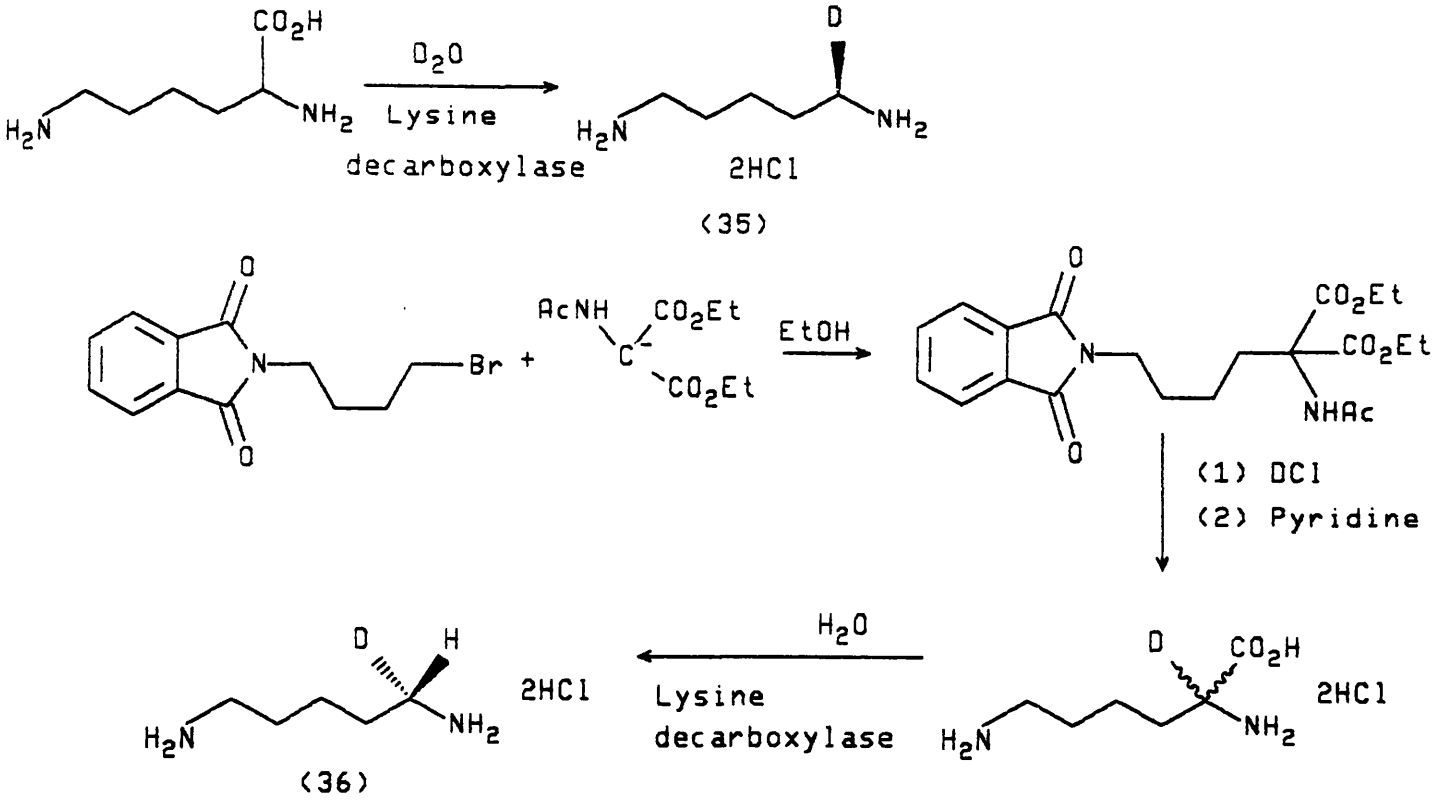
(ii) Stereochemistry of the Processes in the Biosynthetic Pathway

On examination of the biosynthetic pathway to lupinine (4), several stereochemical questions arise. The likely route to lupinine is shown below, with the dissymmetric dimeric intermediate present (Scheme 11). In step (c), the stereochemistry of the ring junction is determined. The stereochemistry of the process is inferred by reference to the known stereochemistry of the final product. This is relatively simple. However, other steps e.g. (b) involve the loss or gain of a proton from a prochiral centre. The stereochemistry of these steps was determined using chirally deuteriated precursors.

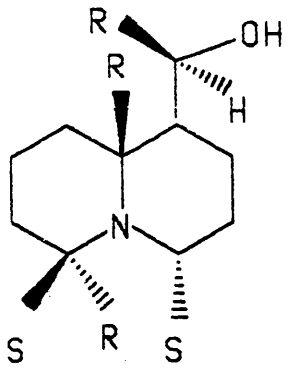
Richards and Spenser prepared (R)- and (S)-[1-^{2}H]-cadaverine dihydrochloride, (35) and (36) respectively, by the enzymic routes shown in Scheme 12.[48] The enantiomeric cadaverines were fed to L. luteus in the usual manner, and the lupinine was extracted and purified as before. The sites of deuteriation were determined by ^{2}H n.m.r. spectroscopy, since the assignments had already been published, this was relatively easy. The labelling pattern discovered is shown in (37).[49]


NH2
CO2H
NH2
(a)
HR
NH2
HS
NH2
(b)
O
H
NH2
H
NH
+
NH
H
H
(c)
NH
NH
+
H
CHO
H
HR
NH
HS
NH2
(d)
H
CHO
si
N
+
re
H
(e)
OH
HR
H
HS
N
HS
HR
HR
HS
(f)
H
CHO
N
HR
HS

Scheme 11[36]


CO2H
D2O
Lysine
decarboxylase
H2N
NH2
D
2HCl
(35)
O
N
Br
+
AcNH
CO2Et
C
CO2Et
EtOH
NHAc
(1) DCl
(2) Pyridine
H2O
Lysine
decarboxylase
H
(36)

Scheme 12



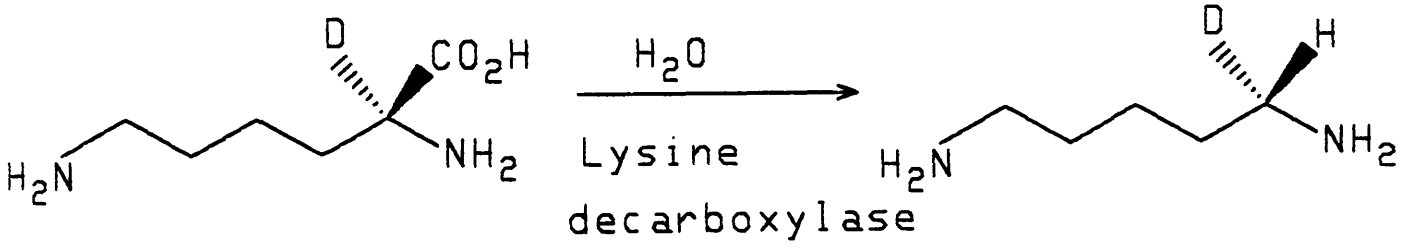
(37)

R denotes ^{2}H present after feeding (R)-[1-^{2}H]-cadaverine hydrochloride. S denotes ^{2}H present after feeding (S)-[1-^{2}H]-cadaverine dihydrochloride. The stereochemistry of the conversions which involve protons on carbon atoms next to the nitrogen and the carbon atom of the carbinol can be deduced from the data. In step (b), the conversion of the diamine to the aminoaldehyde, it is the pro S proton which is lost. This is the expected stereochemistry if the transformation is catalyzed by a plant diamine oxidase or by an L-amino-acid transferase. The entry of the hydride in the reduction step of C=N or $C=\overset{+}{N}$, step (e), takes place from the si face while the entry of the hydride in the reduction of the aldehyde, step (f), occurs from the re face. The fact that both samples of lupinine show deuterium labelling with retention of configuration at C-6 verifies that the C-N bond at this position remains intact. (Loss of N would be expected to result in loss of either or both of the adjacent protons as well).

Two questions concerning the decarboxylation of lysine to cadaverine, step (a), have also been answered by using labelled precursors. It was demonstrated by Golebiewski and Spenser that it is only the L-isomer of lysine that is a precursor of lupinine, using

doubly ^{3}H, ^{14}C-labelled samples of lysine. A sample of L-[4-^{3}H]-lysine with DL-[6-^{14}C]-lysine ($^{3}H/^{14}C$ ratio 4.1 ± 0.1) was administered to L. luteus. The lupinine thus produced had a $^{3}H/^{14}C$ ratio of 8.4 ± 0.1, which corresponds to L-lysine being the precursor.[35]

The same workers proved furthermore that the decarboxylation of L-lysine proceeds with retention of configuration. When DL-[2-^{2}H]-lysine was fed to L. luteus the labelling pattern obtained was identical to that obtained after feeding (S)-[1-^{2}H]-cadaverine (36). Hence the L-lysine enters lupinine via (S)-[1-^{2}H]-cadaverine, and thus the replacement of the carboxyl group by a proton must take place with retention of configuration (Scheme 13). This is in agreement with the known stereochemistry of other amino-acid decarboxylases.[48] The stereochemical results were confirmed by Robins and co-workers.



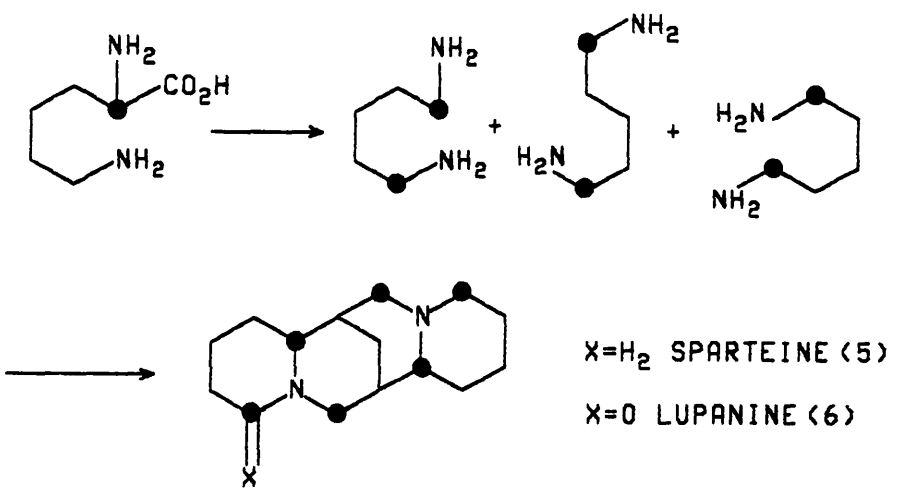
Scheme 13

In conclusion, the use of stable isotopes with ^{13}C and ^{2}H n.m.r. spectroscopy have provided information about the biosynthesis of lupinine far beyond that which was possible using radioactive tracer methods.

2.3 The Biosynthesis of Sparteine and Lupanine-Tetracyclic Quinolizidine Alkaloids

(i) Regiochemistry of Biosynthesis

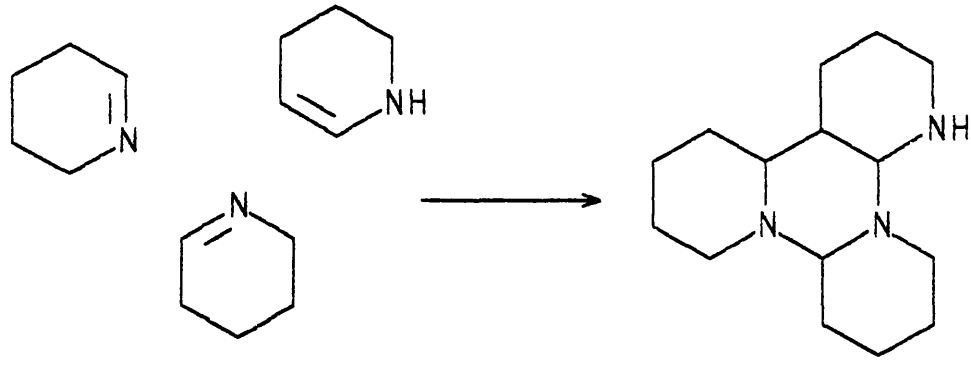
Schütte and co-workers carried out the first tracer experiments on the bisquinolizidine lupine alkaloids, such as sparteine (5) and lupanine (6).[50] Their results, after feeding [2-^{14}C]-lysine, substantiated the view of Sir Robert Robinson that the alkaloids originated from three C_5 chains derived from lysine or cadaverine,[33] and that the nitrogen atoms in the alkaloids also derived from these precursors (Scheme 14). This route did not account for mechanism or stereochemistry.



Scheme 14

In 1976, Spenser put forward an hypothesis for the conversion of cadaverine into the $C_{15}N_2$ alkaloids which accounted for their stereochemistry.[51] This hypothesis viewed the $C_{15}N_2$ lupin alkaloids as

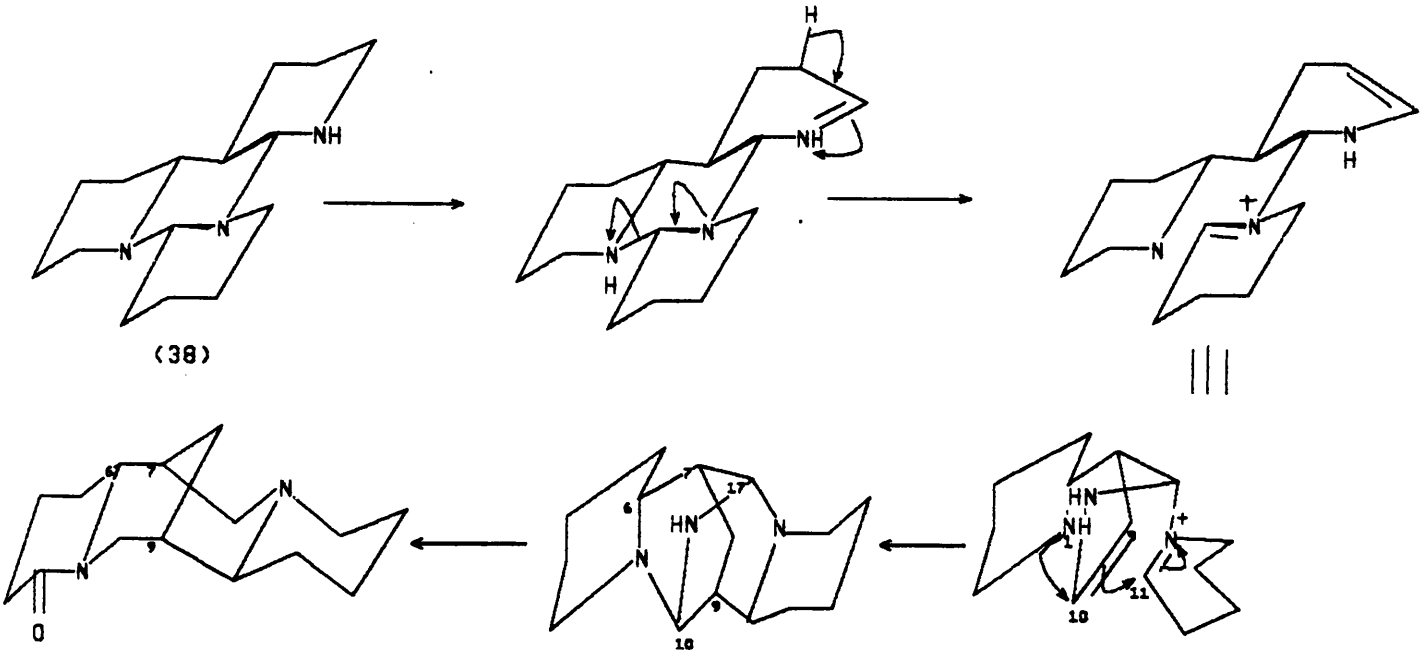
modified trimers of Δ'-piperideine (20) formed from cadaverine by oxidative deamination or by transamination. The trimer is isotripiperideine, and the all-trans (6β, 7α, 11β, 17β) stereoisomer (38) is favoured (Scheme 15).



(38)

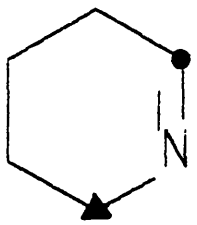
Scheme 15

The stereochemistry of the alkaloids was postulated to be a result of the involvement of the favoured all-trans stereoisomer of isotripiperideine (38) in a four step sequence (Scheme 16). The stereochemistry of the 'prealkaloid' trimer (39) at three sites, C-6, C-7, and C-9, is determined by the stereochemistry of the trimer of Δ'-piperideine from which it originated. The stereochemistry at C-11 however is determined by the course of ring closure, that is whether the intramolecular attack at C-11 is on the re or si face. Golebiewski and Spenser tested this hypothesis by feeding [2-^{14}C]- and [6-^{14}C]-Δ'-piperideine, (40) and (41), respectively, by the wick method to Lupinus angustifolius plants.



(38) (39)

Scheme 16



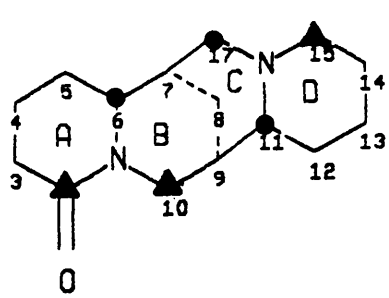
● [2-^{14}C] (40)

▲ [6-^{14}C] (41)

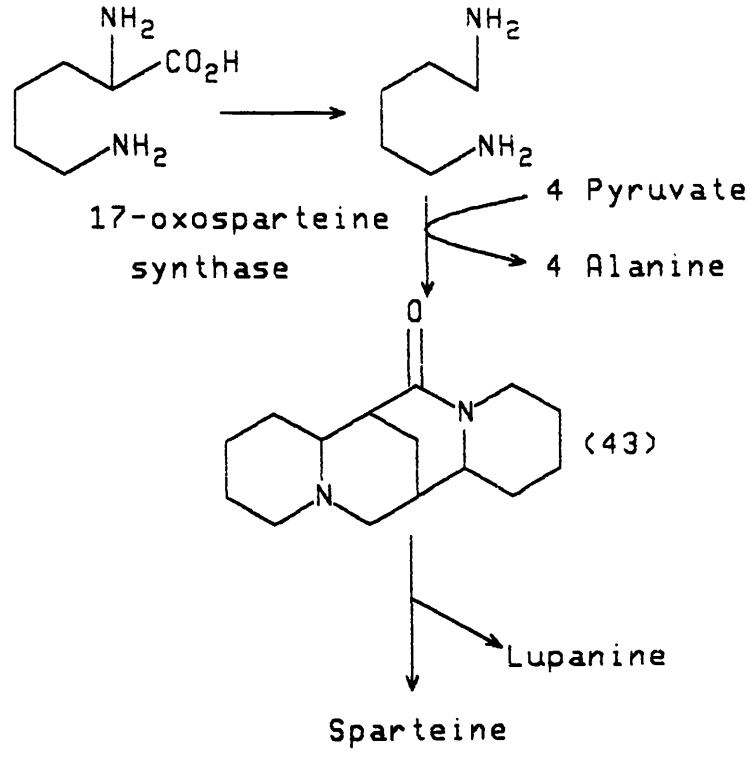
The lupanine was isolated after 3 days and degradation studies were carried out on the material. The observed pattern of labelling is consistent with the alkaloids being derived from Δ'-piperideine via isotripiperideine. Carbon-2 of Δ'-piperideine supplies C-17, C-11 and by inference, C-6 of lupanine, while carbon-6 of Δ'-piperideine supplies C-2, C-15 and again, by inference, C-10 of lupanine (42).[51]

In 1979, Wink and co-workers proposed a different biosynthetic route, based on results from cell suspension cultures of L. polyphyllus.[52] A crude enzyme preparation from these cultures catalyzed the pyruvate-dependent conversion of cadaverine into the tetracyclic lupin alkaloids, the first product always being 17-oxosparteine (43). If the Spenser hypothesis is correct, free molecules of 5-aminopentanal and its cyclised products, e.g. Δ'-piperideine and tetrahydroanabasine should be expected in GC-MS analysis - no free intermediates were observed, however.

Wink and Hartmann postulated that a cadaverine-pyruvate transaminating system, known as "17-oxosparteine synthase", catalyzed the formation of 17-oxosparteine (43) from three cadaverine units without the release of any free intermediates (Scheme 17).



(42)



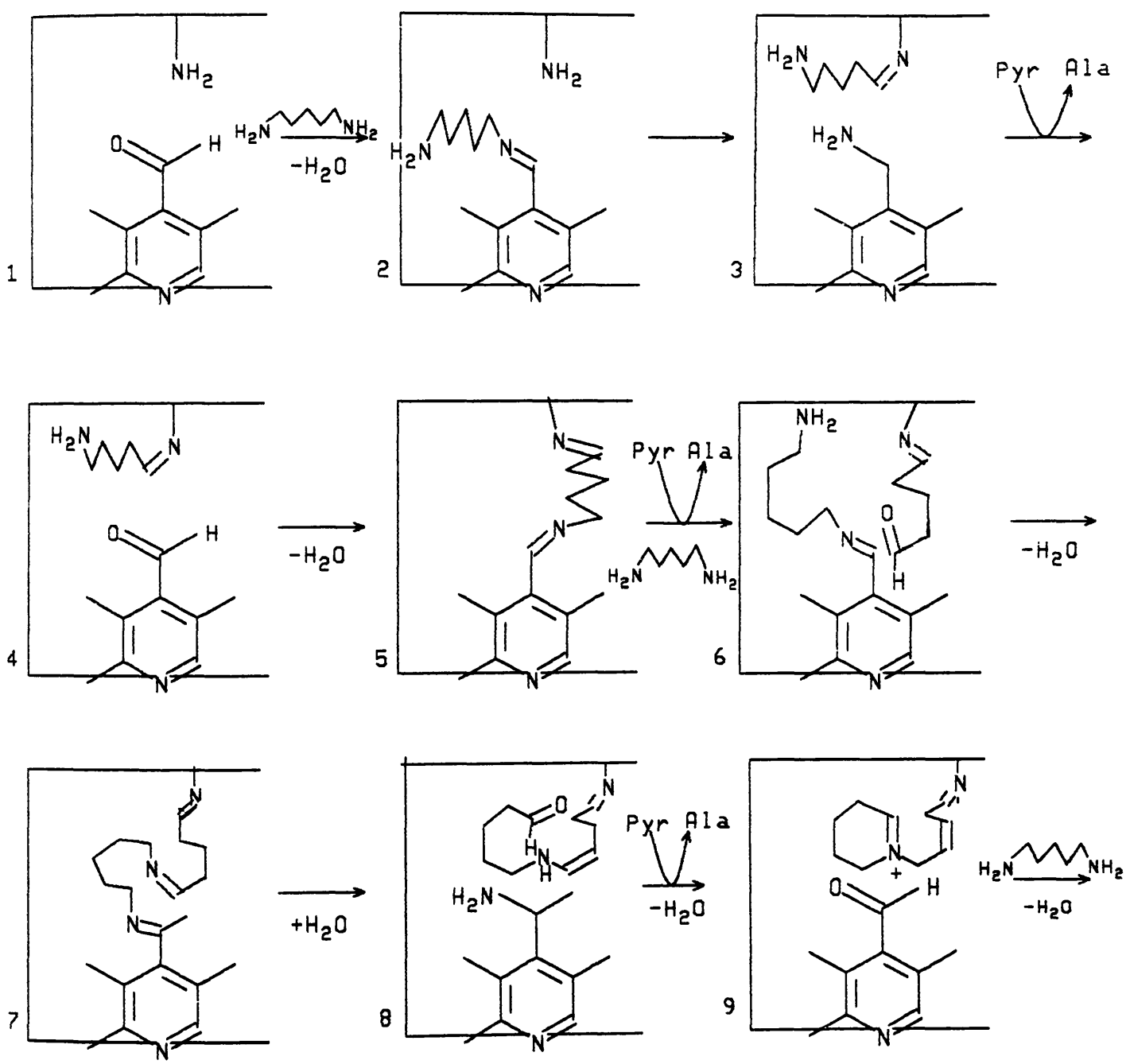
Scheme 17[52]

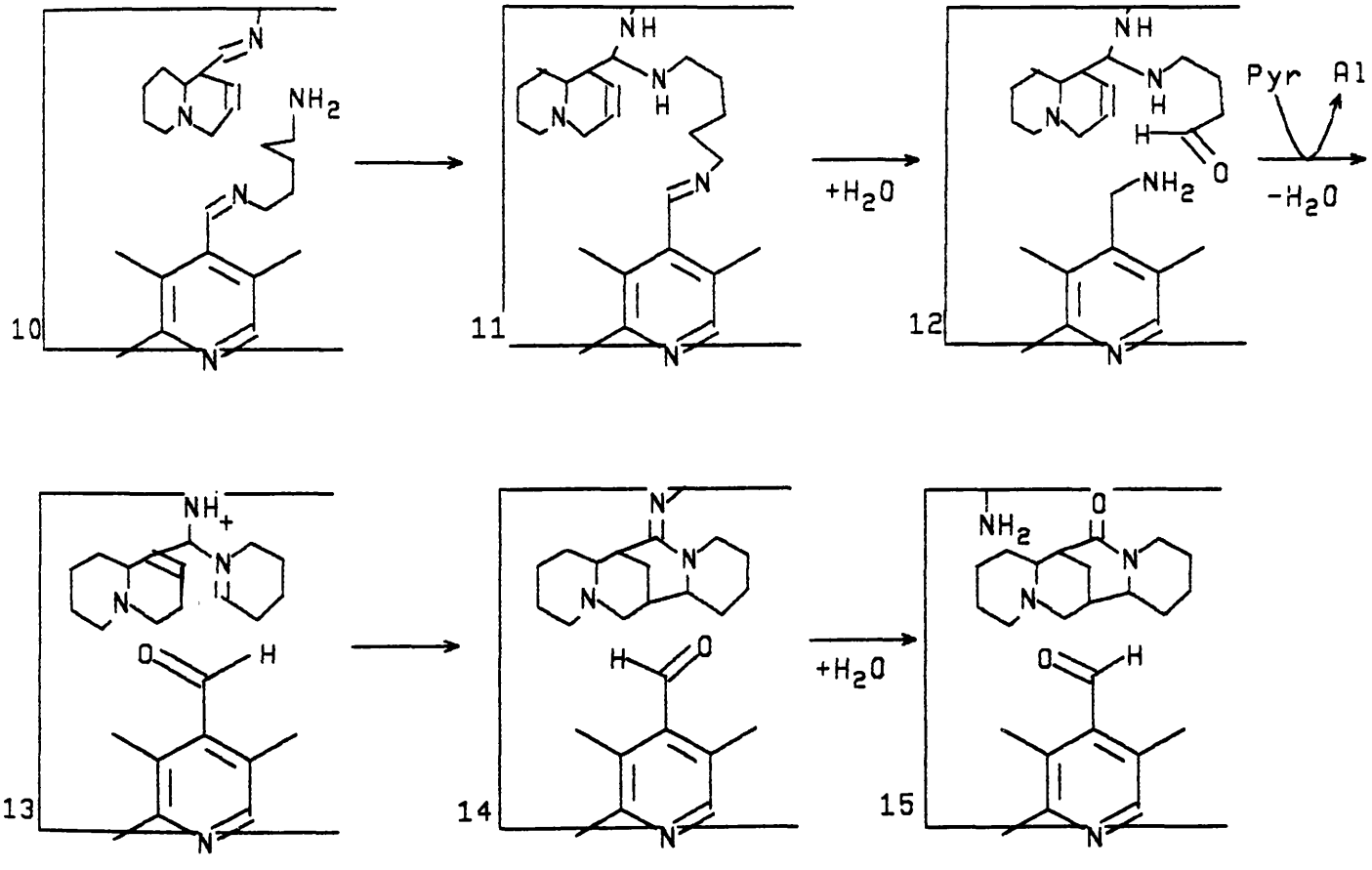
Their model mechanism was designed to account for the results of both the in vivo tracer experiments and the enzymatic studies. A co-enzyme of pyridoxal phosphate with 17-oxosparteine synthase was invoked to account for the four transamination steps involved in the overall reaction. Furthermore, a separate amino group is required for the intermediates to bind to until the final product is released. Scheme 18 shows the mechanism proposed.

The enzymatic synthesis is initiated when the cadaverine binds to the pyridoxal phosphate (Scheme 18/2); the imine thus formed is then transferred to the carrier amino group (Scheme 18/3). The next steps involve transamination of pyruvate , transamination

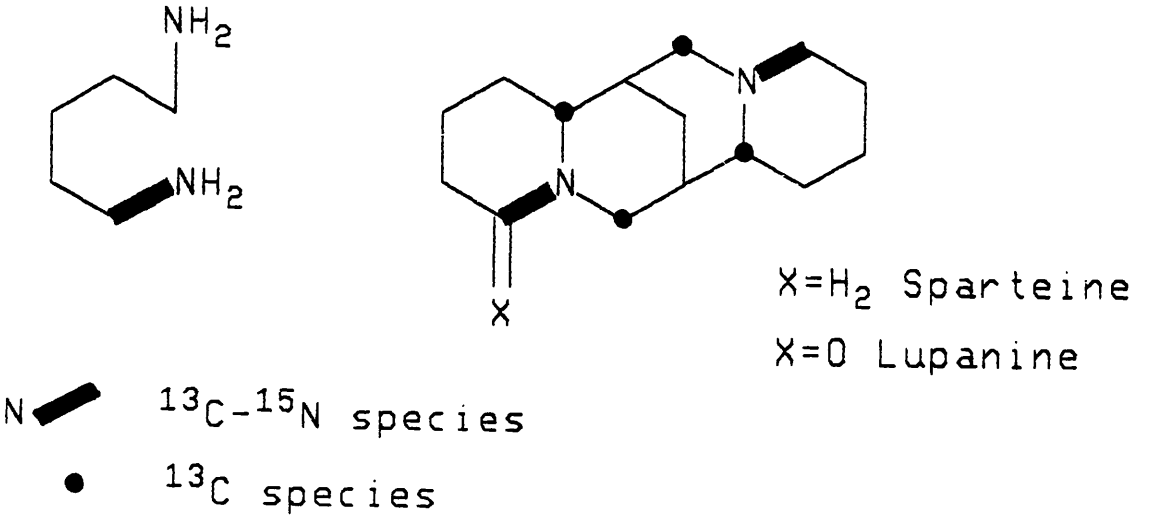
of the second cadaverine unit and condensation of the cadaverine units by Schiffs base formation (Scheme 18/3-7). Steps 8-10 lead, by cyclisation, to the quinolizidine ring system, involving tautomerism and Mannich reaction with formation of a reactive carbonium ion at C-7. After condensation of the third cadaverine unit the cyclisation of the tetracyclic system occurs, involving a similar mechanism as before. After a double bond shift, 17-oxospartine (43) is released, by hydrolysis. Lupinine (4) could be obtained by this mechanism by terminating the process after step 10. The results from Spenser's feeding of Δ'-piperideine were explained by the conversion of Δ'-piperideine (20) to its open-chain form 5-amino-pentanal (19) which can then bind to the enzyme.

In order to determine which C-N bonds remain intact in the formation of tetracyclic quinolizidine alkaloids from cadaverine, Golebiewski and Spenser,[53] and Rana and Robins,[54,55] separately fed [1-amino-^{15}N, 1-^{13}C]-cadaverine to plants which produce sparteine (5) and lupanine (6). The results were the same for both sets of workers; the six carbon atoms adjacent to the two nitrogen atoms were enriched in ^{13}C, confirming the ^{14}C tracer work. However, only two of the six carbon atoms adjacent to nitrogen in sparteine and lupanine, namely C-2 and C-15, showed ^{13}C n.m.r. spectral signals with intense doublets due to ^{13}C-^{15}N coupling (Scheme 19). These observations were consistent with both the piperideine-trimer and the Wink proposals. Some faults with the Wink hypothesis were found following the completion of studies concerning the stereochemistry of the processes.


NH2
H2N
NH2
-H2O
O
H
N
1
2
3
Pyr Ala
4
5
6
7
8
9
+H2O



Scheme 18[52]

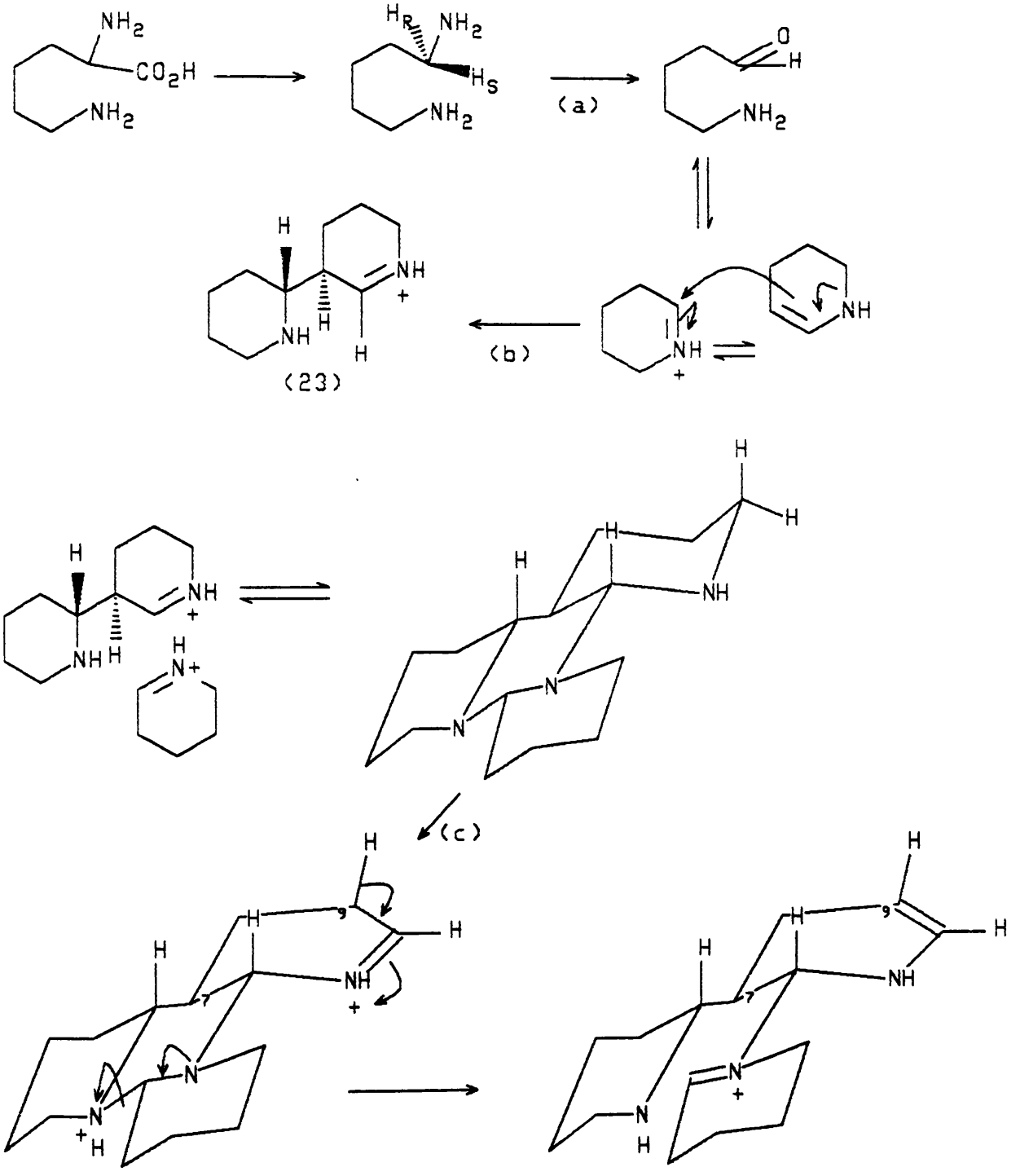


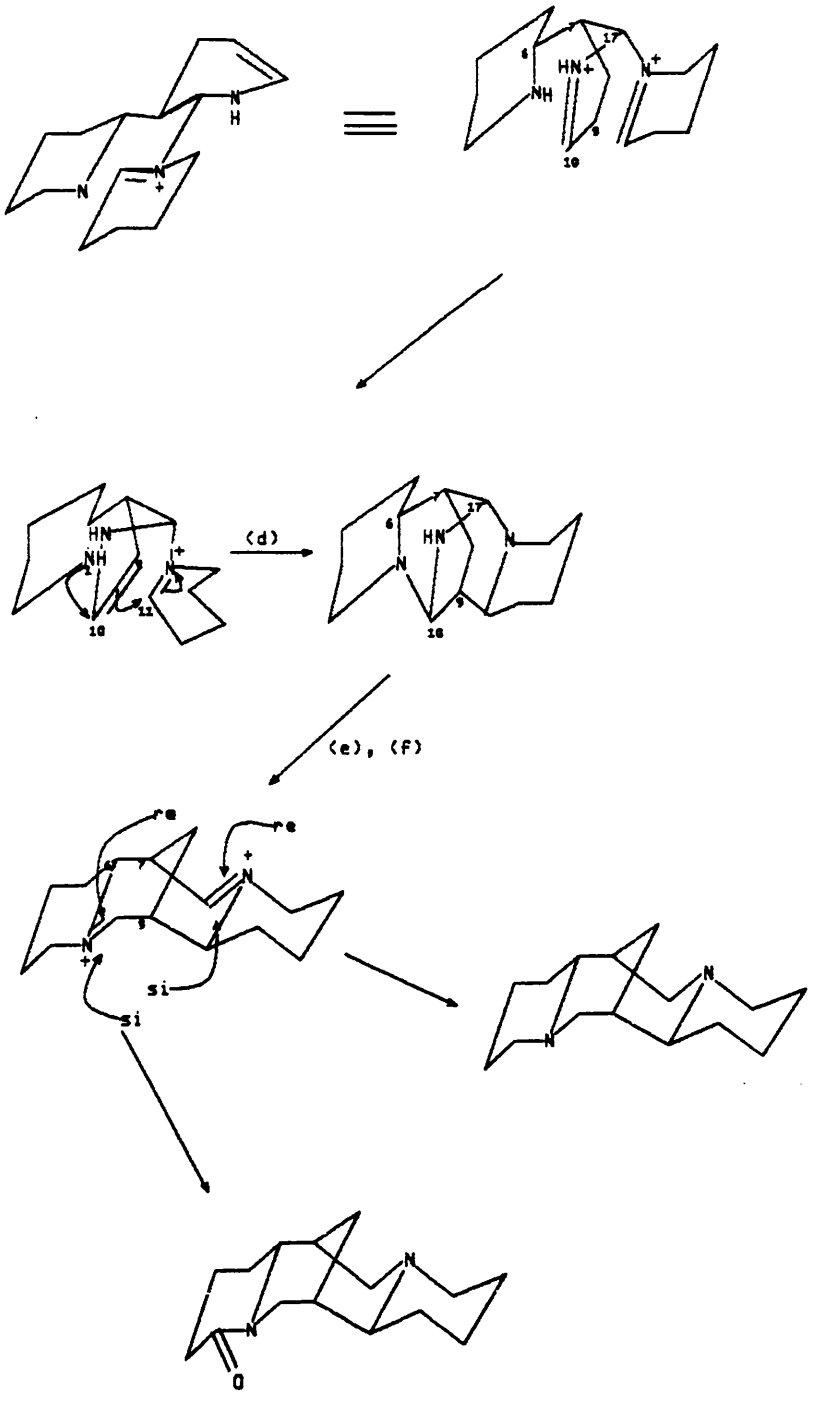
Scheme 19

(ii) Stereochemistry of Biosynthesis

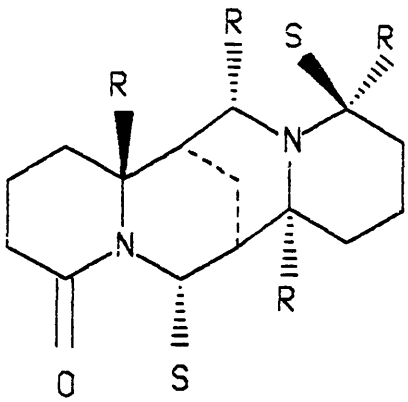
A number of questions about the stereochemistry of the biosynthetic sequence arise on examination of the alkaloids. The stereochemistry of four chiral centres is determined by two steps [Scheme 20, (b) and (d)] and can be ascertained by reference to the known chirality of the final products at C-6, C-7, C-9 and C-11. Other steps, as in the lupinine (4) case, require additional experiments using enantiomerically deuteriated substrates to unlock the hidden stereochemistry.

Golebiewski and Spenser,[53] and Fraser and Robins[56-58] fed (R)- and (S)-[1-^{2}H]-cadaverines to plants producing sparteine and lupanine. The ^{2}H n.m.r. spectra were assigned from published data with the results shown below in (44) and (45).

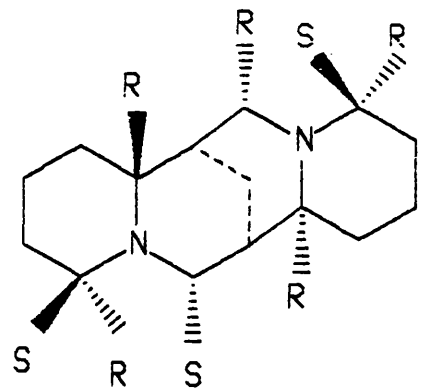

(a)
(b)
(23)
(c)



Scheme 20[36]



(44)



(45)

The labelling patterns reveal the stereochemistry of each of the biosynthetic steps that involve prochiral sites adjacent to nitrogen. As is the case in the formation of lupinine, cadaverine is converted into the aminoaldehyde with loss of the pro S proton from C-1. Again, the entry of hydride in the reduction of C=N bonds occurs from the C-re face [steps (e) and (f)] as in lupinine. In step (c), it is the pro R proton, from the carbon atom which becomes C-10, which is lost.

The results from these experiments not only establish the stereochemistry of the biosynthetic steps of the pathway; they also provide essential evidence against the feasibility of some proposed theories.

Since both lupanine (6) and sparteine (5) retain deuterium from R-[1-^{2}H]-cadaverine at the 17α position, with an enrichment similar to positions 11α and 6β, 17-oxosparteine cannot be a precursor of lupanine or sparteine. The pathway proposed by Wink, and discussed earlier, is thus invalidated at least in the later stages.

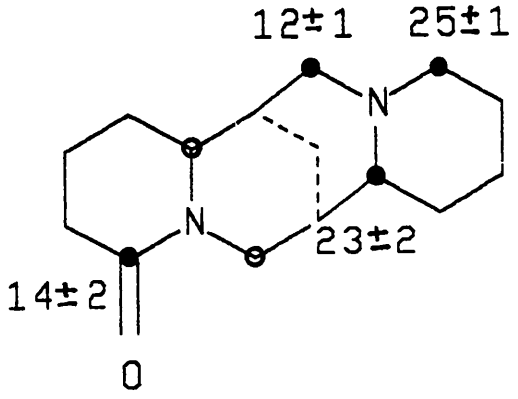
Furthermore, since the samples retain deuterium from (S)-[1-^{2}H]-cadaverine at 10α, a suggestion by Spenser that 10-oxosparteine

may be an intermediate is also not correct.

It was suggested that sparteine originates by reduction of lupanine, or by reduction of the 1,2-dehydrospartenium ion. Since deuterium is retained by sparteine at both 2α and 2β positions from (R)-[1-^{2}H]-cadaverine and (S)-[1-^{2}H]-cadaverine respectively, these proposals are disproved.

The Δ'-piperideine trimer route for the biosynthesis of sparteine and lupanine, however, remains consistent with all the evidence so far.

Recently, Golebiewski and Spenser proposed a new biogenetic model, based on observations after feeding DL-[6-^{14}C]-lysine and [6-^{14}C]-Δ'-piperideine to L. angustifolius.[59] The lupanine extracted showed a higher level of incorporation into one of the C_5 segments, while levels in the other two C_5 units were identical. The distribution of label was established by chemical degradations of lupanine (46). For the DL-[6-^{14}C]-lysine feed, one quarter of the total activity of the alkaloid was located at each of C-15 and C-11, so that the C_5N unit, N-16, C-15, -14,-13,-12,-11 accounted for half the activity within the lupanine molecule. The other half of the activity was spread over the rest of the molecule, C-2 and C-17, with each containing one eighth of the total activity, C-6 and C-10 presumably containing the other two eighths.



(46)

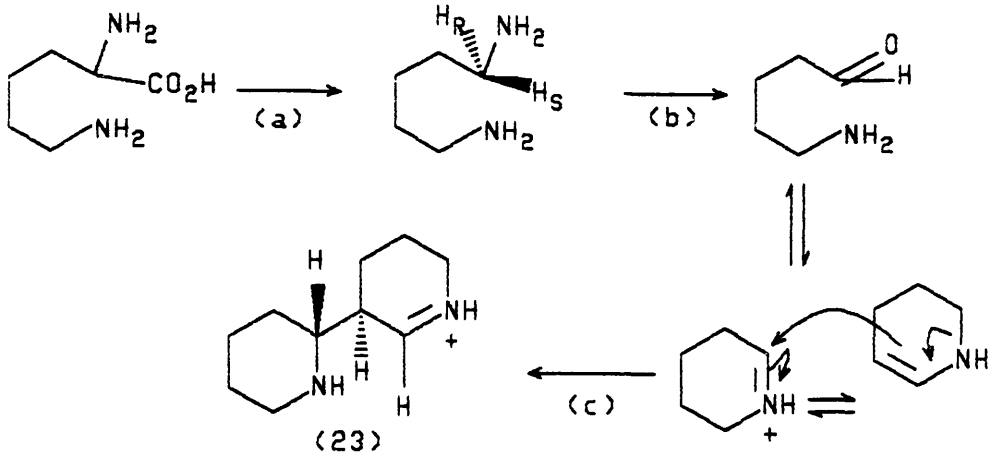
This distribution of label cannot arise if three C_5N monomers simultaneously form a trimer on their way to lupanine. In such a scenario, equal distribution of ^{14}C label would be expected in all three C_5N segments. One of the three identical C_5N units, that which becomes the C_5N moiety N-16, C-15,-14,-13,-12,-11 of the alkaloids, must experience less endogenous carrier dilution than the other two units on route to the product.

Golebiewski and Spenser proposed a modifed biosynthetic model, which is consistent with the new findings and also accommodates the earlier results (Scheme 21). The two key intermediates are the Δ'-piperideine dimer, tetrahydroanabasine (23) and the bisquinolizidine derivative 1,10; 16,17-didehydrosparteinium ion (47). Stage A of the new route is identical to steps on the route to lupinine, discussed earlier.

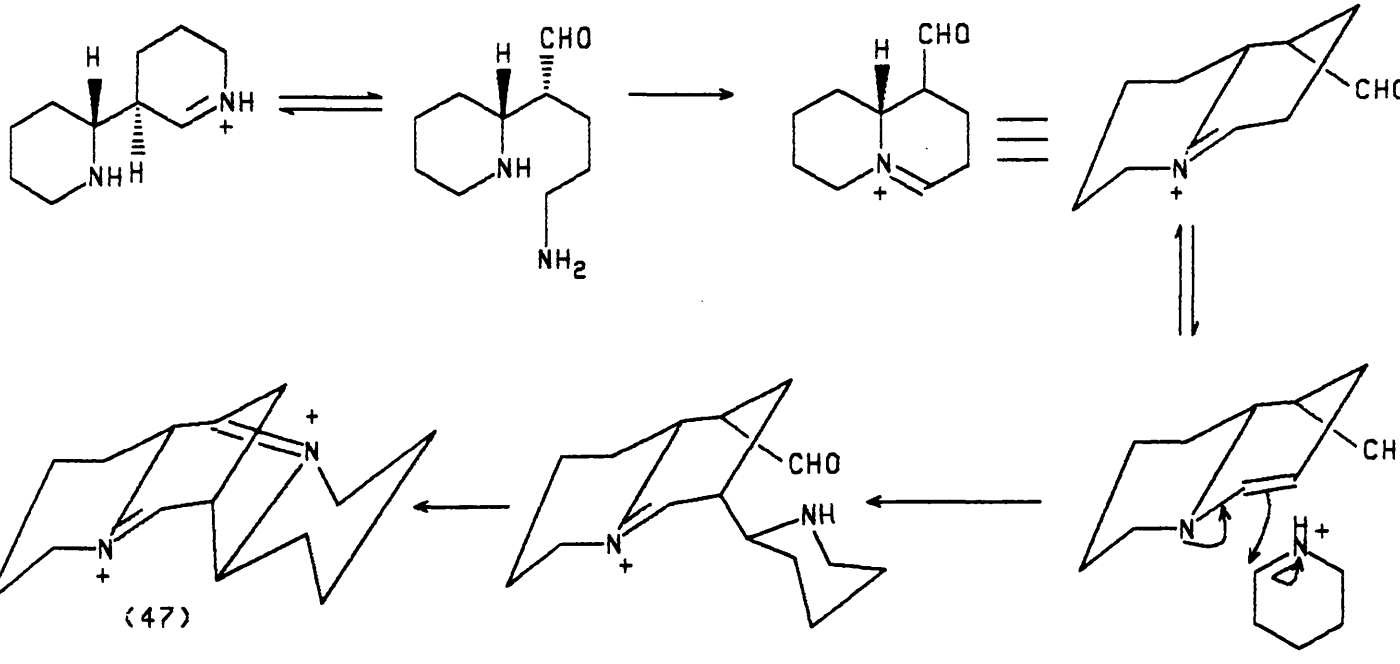
The intermediate steps of the route, from tetrahydroanabasine (23) to didehydrosparteinium ion (47) are possible by two different routes which vary in the timing of the loss of nitrogen from the C_5N precursor unit that becomes the central C_5N chain, C-10,-9,-8,-7,-17 of the bisquinolizidine skeleton. In Variant I, nitrogen is eliminated from the $C_{10}N_2$ tetrahydroanabasine-derived intermediate, which also serves as a building block for lupinine. After nitrogen loss the intermediate condenses with another C_5N unit, to form eventually the didehydrosparteinium ion (47).

Variant II involves a modified Δ'-piperideine trimer and implies loss of nitrogen at a later stage. Both variants predict that the two C_5 chains (C-2,-3,-4,-5,-6 and C-10,-9,-8,-7,-17) are derived from tetrahydroanabasine (23), which is the dimer of Δ'-piperideine. Hence the

Stage A

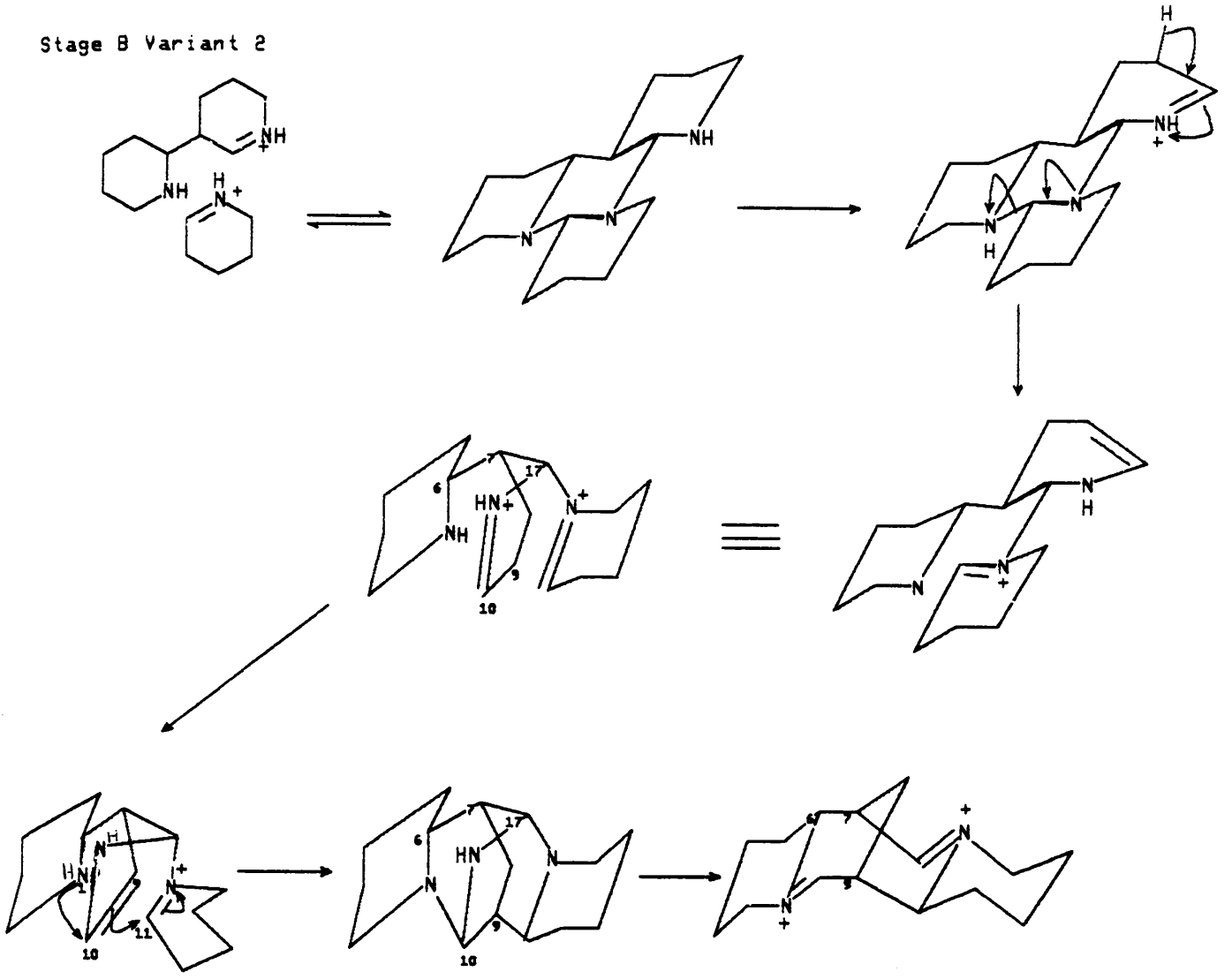


Stage B Variant 1



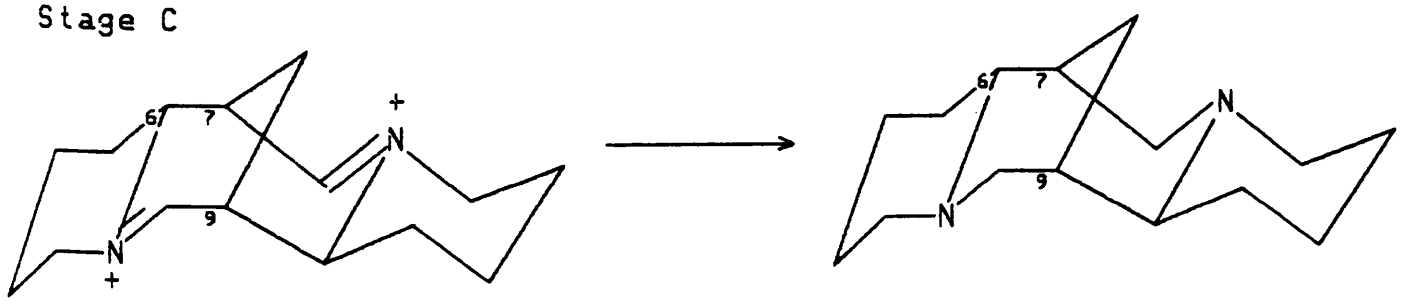
Scheme 21a

Stage B Variant 2



Scheme 21B

Stage C



Scheme 21c

four carbon atoms C-2,-6,-10 and -17 of lupanine and sparteine should be equally enriched in ^{14}C when ^{14}C-lysine is fed. In addition, the C_5 chain C-11,-12,-13,-14,-15 originates from a third Δ^1-piperideine unit that condenses with the previously formed C_{10} intermediate in a later, separate step. Since the pool sizes of the C_{10} and C_5 intermediates are not necessarily the same in the intact plant, conditions in a labelling experiment may be such that the delivery of the C_5 chain C-11,-12,-13,-14,-15 is of a different dilution to that of the C_{10} unit C-2,-3,-4,-5,-6;C-10,-9,-8,-7,-17. In stage C, the alkaloids are formed from the 1,10;16,17-didehydrosparteinium ion (47).

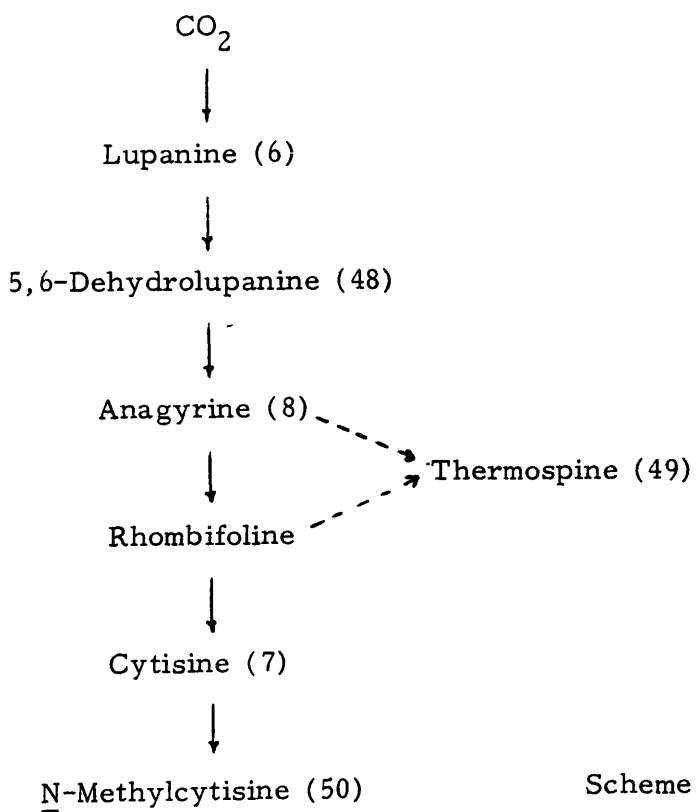
According to Golebiewski and Spenser, this hypothesis opens the door to new investigations: nevertheless the fundamental issue of whether or not Δ^1-piperideine is a direct intermediate of the quinolizidine alkaloids is not yet resolved.

Recently, Perrey and Wink administered ^{14}C-labelled Δ^1-piperideine and α-tripiperideine (38), as well as cadaverine, to leaf discs of L. polyphyllus, in short term experiments of 12-16 hour duration.[60] The cadaverine was incorporated in good yields into lupanine, but Δ^1-piperideine and tripiperideine were incorporated between 7 and 60 times less than cadaverine. The results suggest that although these latter compounds are incorporated to a certain degree, they are not direct intermediates of lupanine and sparteine.

It is evident from the preceding discussion that this area of research is still the subject of much controversial debate.

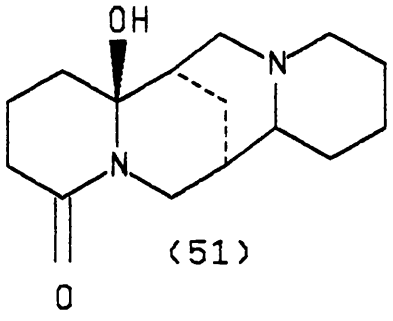
2.4 Biosynthesis of Pyridone Quinolizidine Alkaloids

Cho and Martin studied the biosynthesis of Thermopsis alkaloids from $^{14}CO_2$ in order to determine the chronology of appearance of ^{14}C in a mixture of quinolizidine alkaloids, and thus to gain an insight into the origin of the pyridone alkaloids.[61] They found that lupanine (6) appeared as the earliest, most-rapidly formed alkaloid, reaching a maximal specific activity between 2 to 4 hours and then decreasing, while the other alkaloids were increasing in activity. This is consistent with a rapid turnover or transformation into the other alkaloids. The observed chronological order of formation of the alkaloids is shown in Scheme 22.



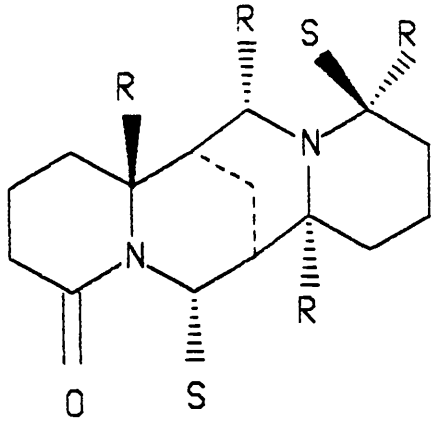
Scheme 22

They proposed that the pyridone bases are derived from lupanine via 5,6-dehydrolupanine (48).[62] Recently, the absolute configuration of 5,6-dehydrolupanine has been determined[63] showing that it is the same as that of (-)-lupanine (6) and (-)-anagyrine (8). These findings make it more plausible that (48) is the biosynthetic intermediate between (6) and (8). Also, 6β-hydroxylupanine (51) has been isolated by Asres and co-workers, and may be an intermediate between lupanine and 5,6-dehydrolupanine.[64]

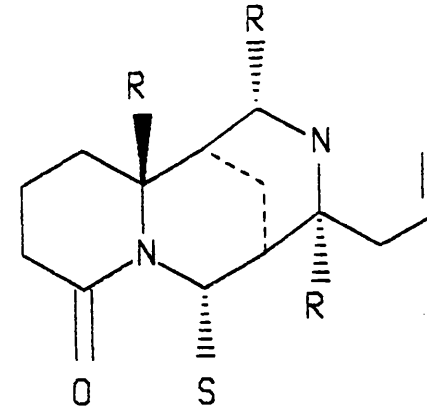


(51)

Evidence produced from stereochemical studies on the pyridone alkaloids is, however, in conflict with some of these proposals. Lupanine (6) and angustifoline (9) are both found in L. polyphyllus providing circumstantial evidence that it is ring D of a tetracyclic precursor that is cleaved to yield the tricyclic alkaloid, and that ring A is oxidised to the lactam. Feeding experiments with (R)- and (S)-[1-^{2}H]-cadaverines[56] gave the labelling patterns shown below supporting the theory [(52) and (53)].

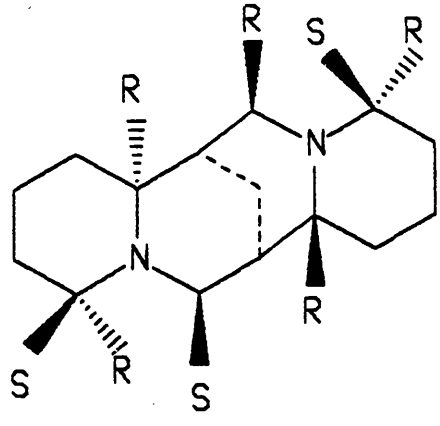


(52)

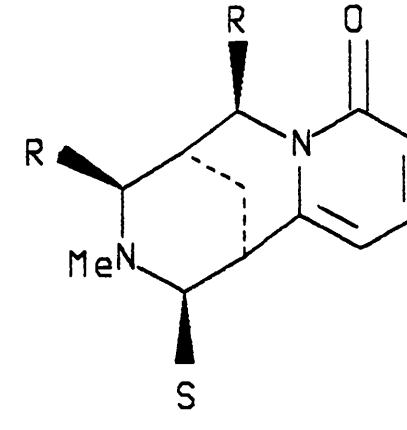


(53)

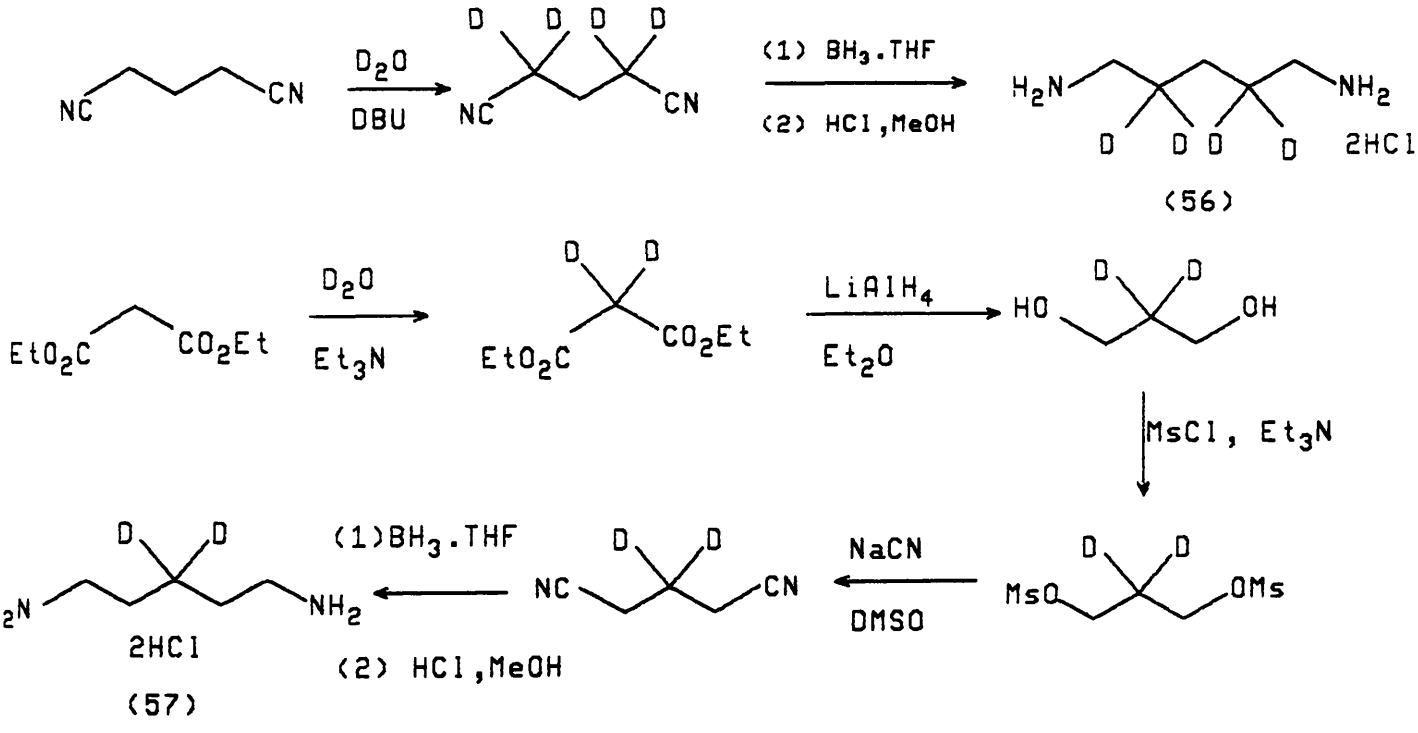
By analogy, it was suggested that ring D is cleaved and ring A becomes a pyridone to form the tricyclic alkaloid, N-methyl cytisine (50). However, when plants of Baptisia australis, which produce (+)-sparteine (5), (-)-N-methylcytisine (50) and (-)-cytisine (7) were fed with (R)- and (S)-[1-^{2}H]-cadaverine, the labelling patterns observed (54) and (55) conflicted with this proposal.[57]



(54)



(55)


NC
CN
D2O
DBU
D D D D
NC
CN
(1) BH3.THF
(2) HCl,MeOH
H2N
NH2
D D D D
2HCl
(56)
EtO2C
CO2Et
D2O
Et3N
D D
EtO2C
CO2Et
LiAlH4
Et2O
HO
OH
D D
MsCl, Et3N
MsO
OMs
D D
NaCN
DMSO
NC
CN
D D
(1)BH3.THF
(2) HCl,MeOH
H2N
NH2
D D
2HCl
(57)

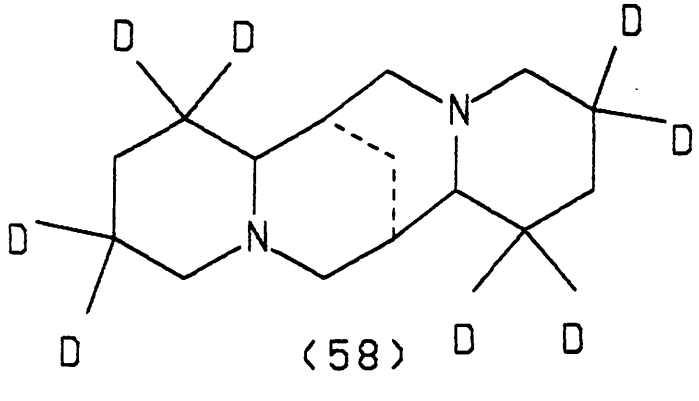
Scheme 23

If (+)-sparteine and (-)-N-methylcytisine are derived from the same tetracyclic precursor, then ring A must be degraded and ring D must be converted into a pyridone to produce the observed signals at C-10 and C-13. The ^2H label at C-11 of (-)-N-methylcytisine (50) derived from the (R)-precursor is retained on cleavage of ring A, but its stereochemistry is inverted. This can be explained by invoking a C-11,N-12 immonium ion intermediate which is reduced stereospecifically from the re-face. Because the N-methyl group has no ^2H labels it cannot be derived from C-2 of a tetracyclic precursor. It has been demonstrated that (-)-N-methylcytisine (50) is formed from (-)-cytisine (7) by an N-methyltransferase enzyme, thus supporting this finding.

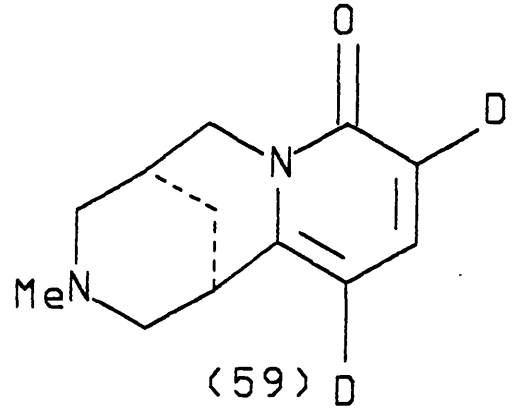
Robins and Sheldrake fed [2,2,4,4-$^2H_4$]-cadaverine (56) and [3,3-$^2H_2$]-cadaverine (57) to Baptisia australis.[65] The cadaverines were synthesised as shown in Scheme 23.

Each precursor was fed to B. australis, and the alkaloids (+)-sparteine (4), (-)-cytisine (7) and (-)-N-methylcytisine (50) were separated by column chromatography. The labelling patterns observed are shown below.

[2,2,4,4-$^2H_4$]-cadaverine feed

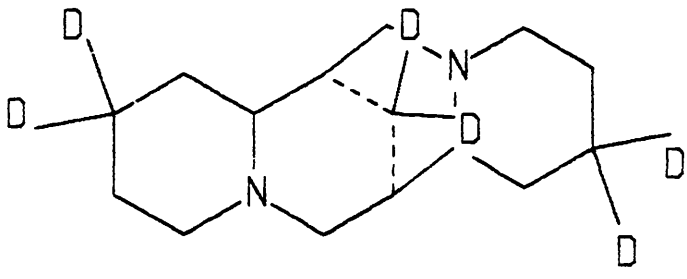


(58)

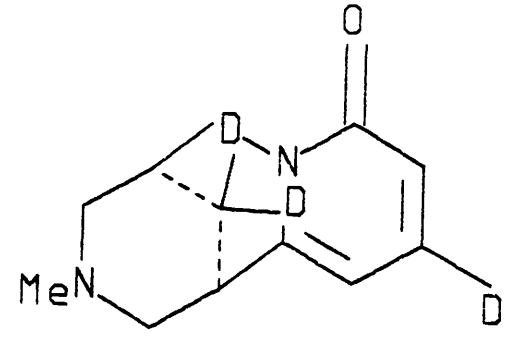


(59)

[3,3-$^2H_2$]-cadaverine feed



(60)



(61)

The presence of the ^2H labels in the pyridone ring of (-)-N-methylcytisine shows that there are no keto or enol intermediates involved in the formation of the pyridone at these carbon atoms. The absence of ^2H labels at C-7 and C-9 in both (+)-sparteine and (-)-N-methylcytisine suggests that enamine-imine (or immonium ion) equilibria are involved in the biosynthetic pathways to remove ^2H from these positions.

Obviously there is much work still to be done if we are to comprehend fully the way in which pyridone alkaloids are biosynthesised, and their relationship to the tetracyclic alkaloids. Some of this research is described in Chapter 3.

2.5 Enzymology of the Biosynthesis of Quinolizidine Alkaloids

The enzymatic approach used by Zenk, Stöckigt, Reuffer and co-workers on the biosynthesis of indole and isoquinoline alkaloids has been very successful,[66] due to the improvement of biochemical techniques and the availability of suitable experimental systems, such as plant cell suspension cultures. Wink and co-workers have studied the enzymology

of quinolizidine alkaloid biosynthesis in plants and cell cultures since 1976.[67]

The enzyme lysine decarboxylase (LD), which converts lysine into cadaverine, has been found in lupin cell cultures and intact plants.[68,69] It is localized in the stroma of chloroplasts, where quinolizidine alkaloid biosynthesis takes place.

Another enzyme, oxosparteine synthase (OS), has been isolated from leaf chloroplasts of L. polyphyllus.[70,71] This enzyme directly converts three units of cadaverine into the tetracyclic alkaloid 17-oxosparteine (43).[72] Since no free intermediates could be directed, an enzyme model with bound intermediates was postulated for the biosynthesis and has been discussed earlier. However, results of tracer studies performed in vivo by the groups of Robins and Spenser disprove the involvement of 17-oxosparteine as an intermediate. There are two plausible explanations for this discrepancy:

(i) The enzyme systems in suspension-culture cells and intact plants are different.

(ii) A number of enzyme activities were present in the cell-free extracts because the enzyme was difficult to purify. Another enzyme may, therefore, have oxidised an intermediate, such as 1,10;16,17-didehydrosparteinium ion (47) to 17-oxosparteine (43).

The product of the oxosparteine synthase reaction and the reaction mechanism are still under study by Wink. The fact that the enzyme, now called oxosparteine synthase exists, however, remains undisputed.

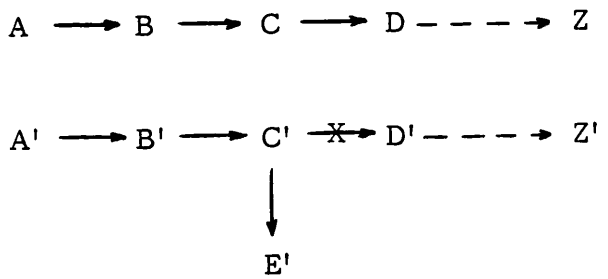
Lysine decarboxylase and oxosparteine synthase show similar activity, have similar partial solubilities and K_m values, and operate best at an alkaline pH. Since free cadaverine is virtually undetectable *in vivo*, the two enzymes must work in a highly co-ordinated manner.

A number of additional enzymes have been isolated and characterised including a tigloyl-CoA:13-hydroxylupanine *O*-tigloyl transferase (THT) which catalyzes the esterification of 13-hydroxylupanine with tiglic acid and an *S*-adenosylmethionine:cytisine *N*-methyltransferase (SAM:ACT).[73,74]

As experimental techniques improve, the enzymatic approach will expand and, hopefully, provide conclusive evidence for the actual route of the biosynthesis of quinolizidine alkaloids.

2.6 The Use of Precursor Analogues in Biosynthetic Studies

It has been recognised for many years that analogues of precursors can be used to study biosynthetic pathways. Living systems may or may not be able to convert the modified precursor into an analogue of the product, giving valuable insight into the enzyme processes occurring in the system. In some cases the metabolism may be changed dramatically, and abnormal growth or even death of the cells may occur. These processes can be represented in a diagrammatic form (Scheme 24).



Scheme 24

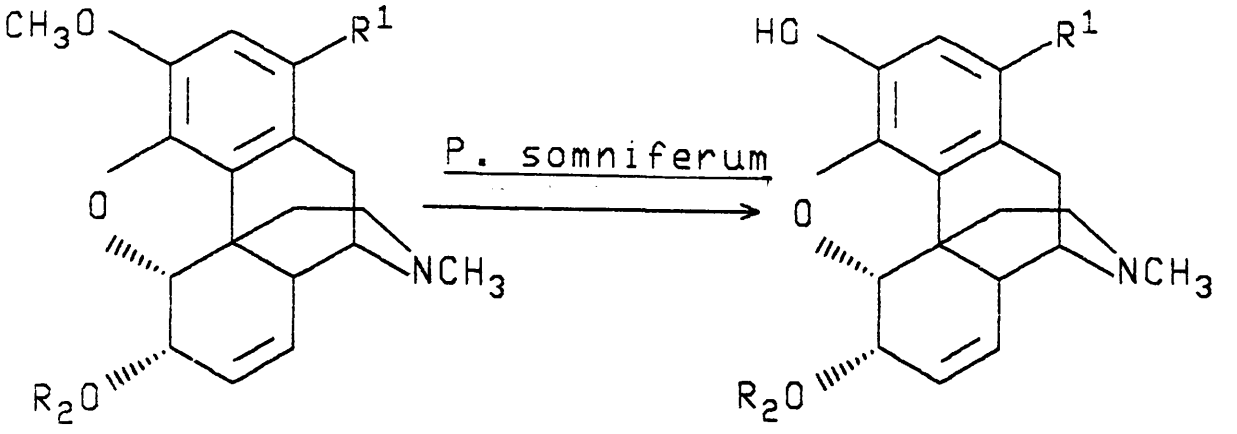
A structurally modified form (A') of a well-defined precursor (A) can be used to construct an analogue (Z') of the final product (Z). Occasionally a biosynthetic step can be retarded e.g. formation of D', which may lead to the accumulation of C' or, alternatively, the system may employ another pathway to produce an intermediate E'. These possibilities are best clarified by means of some examples.

The fundamental feature of an analogue of a known precursor is the replacement of an atom or group, often hydrogen, by a detectable label. The label must have similar properties to the atom it is replacing e.g. Van der Waals radius. Table 1 shows various replacements for hydrogen and their radii.

Atom or Group	Van der Waals Radius (Å)	C-X Bond Energy (kJ/mole)
H	1.20	416
F	1.35	485
Cℓ	1.80	327
Br	1.95	285
CH_3	2.00	256

Table 1

A range of morphine analogues have been isolated from Papaver somniferum after feeding codeine derivatives.[75] Examples of this are the synthesis of 1-bromomorphine (62a) and morphine methyl ether (62b) (Scheme 25).

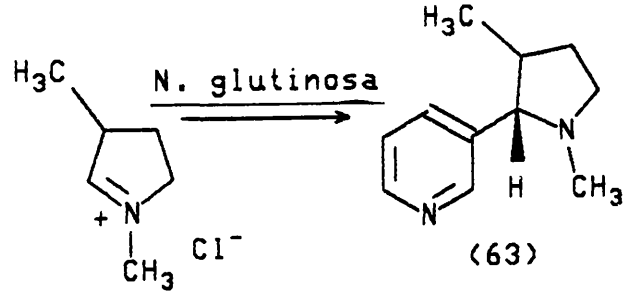


(62a) R^1=Br, R^2=H

(62b) R^1=H, R^2=CH_3

Scheme 25

Administration of 1,3-dimethyl-1-pyrrolinium chloride to Nicotiana glutinosa resulted in the production of an unnatural alkaloid, 3'-methylnicotine (63) (Scheme (26).[76]



(63)

Scheme 26

The vast majority of analogue work, however, has made use of fluorine as the replacement for hydrogen.[77] It has several properties suited to this purpose:

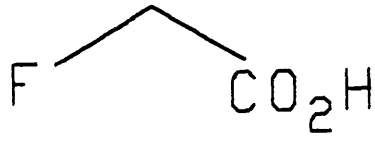
(i) Its Van der Waals radius is comparable to that of hydrogen and so steric interactions with enzymes should be similar.

(ii) It forms a strong bond with carbon, and should not, therefore, be readily displaced.

(iii) It is not common in living organisms and can thus be easily observed by the sensitive technique of ^{19}F n.m.r. spectroscopy.

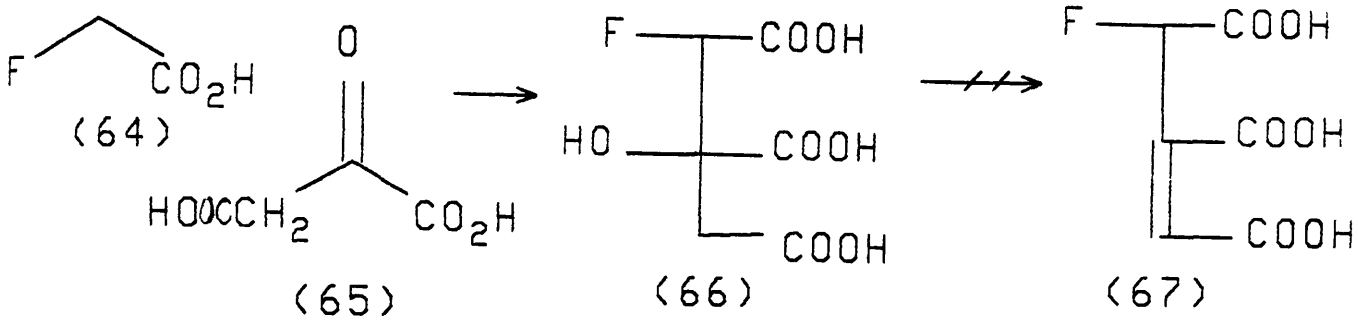
Nevertheless, fluorine is strongly electronegative and if attached to a reaction centre is a moderately good leaving group. It also alters the reaction rates due to its inductive electron-withdrawing effect if in the vicinity of the reaction centre. C-F bonds increase lipophilicity and the CF_3 group is one of the most lipophilic groups known. This increases the compound's solubility in fats, an important consideration in drug design.[78]

The first work on fluorinated compounds in biological systems was concerned with fluoroacetic acid (64), which was isolated by Marais in 1943 from a South African plant Dichapetalum cymosun.[79] This compound proved to be extremely toxic to cattle, and studies were initiated by Peters and Martius.[80,81]



(64)

In biological systems, fluoroacetic acid (64) mimics acetic acid so well it can intrude into the tricarboxylic acid cycle (citric acid cycle). When it is ingested it combines with oxalylacetic acid (65) to give α-fluorocitric acid (66) (Scheme 27).

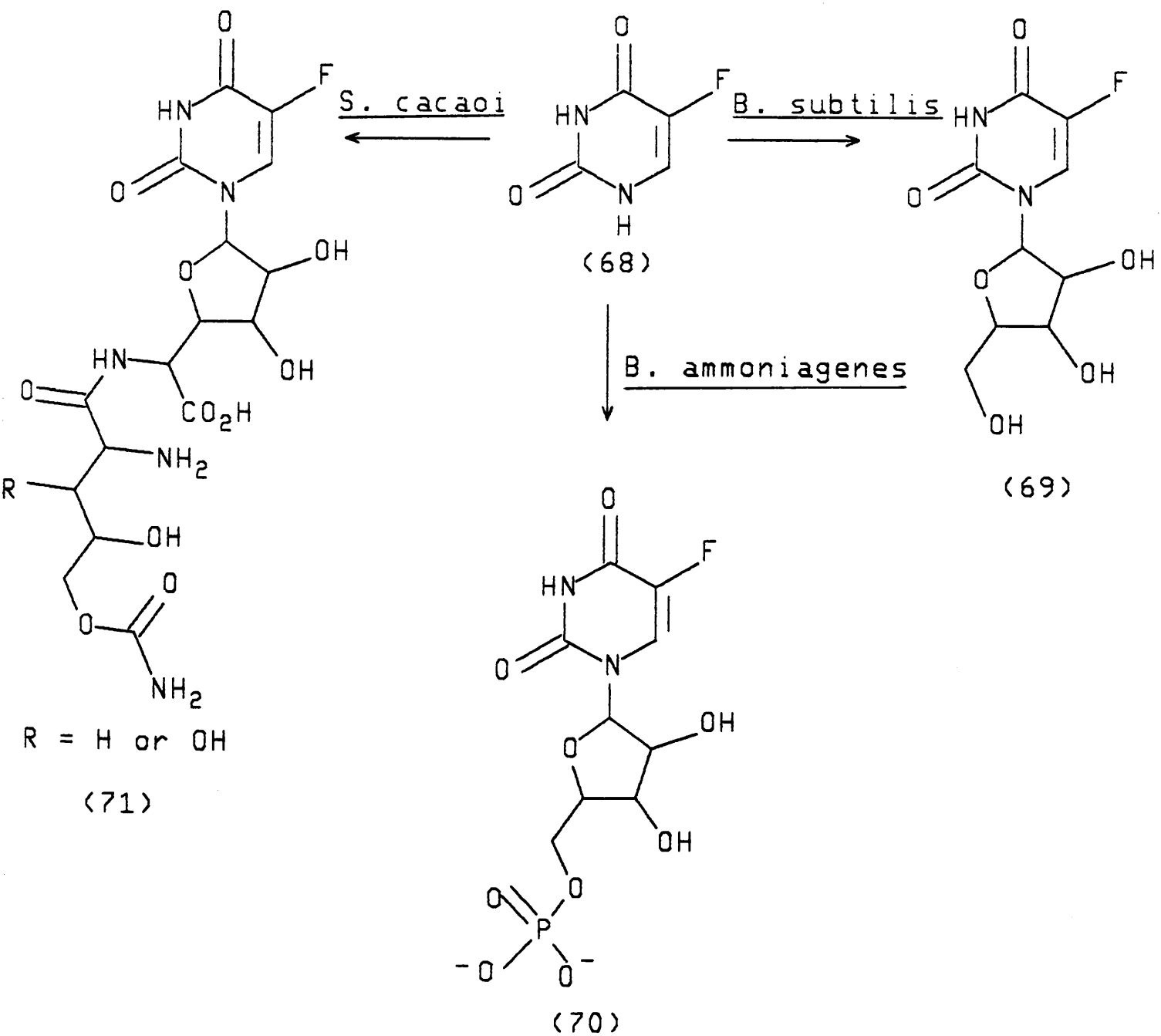


Scheme 27

The next step in the metabolic cycle would be dehydration to aconitic acid (67) but the fluorine atom drastically reduces this process. The fluorocitric acid binds irreversibly to the enzyme, and the metabolism is blocked. Thus the organism has performed a "lethal synthesis" - the incorporated fluoroacetic acid is converted in vivo into an anti-metabolite which blocks the vital tricarboxylic acid cycle.

Once the principle of "antimetabolite formation by introduction of fluorine" had been recognised, applications to chemotherapy were sought. Heidelberger, Duschinsky and co-workers prepared 5-fluoro-uracil (68) and its derivatives.[82] 5-Fluorouracil was found to be incorporated in place of uracil into RNA of bacterial and mammalian cells as well as that of viruses causing mutagenesis, transcription errors and other defects. In Bacillus subtilis, it is converted into 5-fluorouridine

(69), in Brevibacterium ammoniagenes into 5-fluorouridylic acid (70) and in Streptomyces cacaoi (var. asoensis) into 5-fluoropolyoxins (71) (Scheme 28).[83]

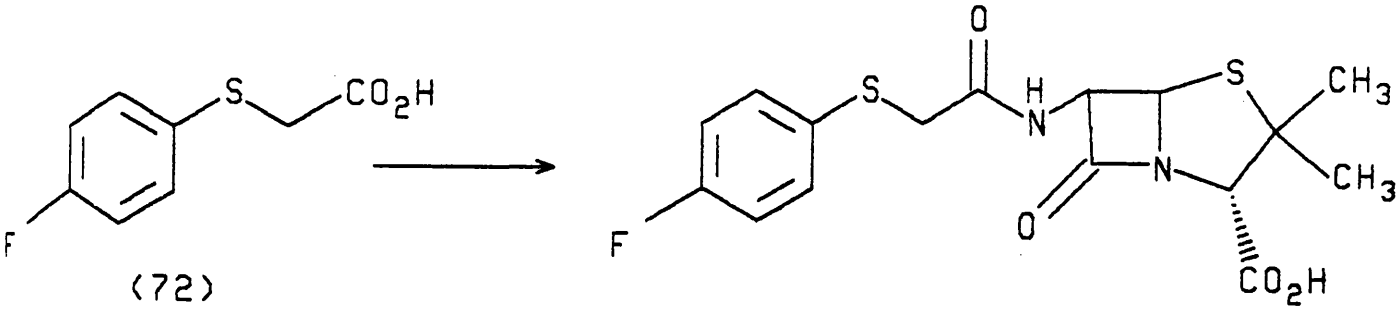


Scheme 28

More importantly, it was shown that 5-fluorouracil blocks the synthesis of thymidylic acid and so thymine is no longer available; the resulting consequences are that replication of DNA can no longer continue, and cell-division is impossible. By this mechanism the inhibitory effect of 5-fluorouracil on tumour growth is explained. This is another example of "lethal synthesis".

Several simple aromatic compounds containing fluorine have been incorporated into analogues of natural products. Some details of these compounds are given below.

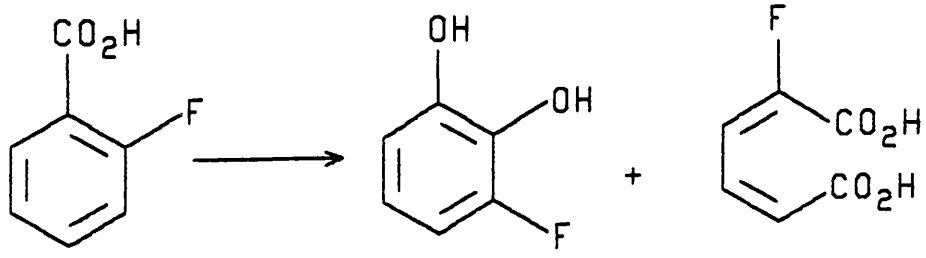
4-Fluorophenylthioacetic acid (72) was used to produce the side-chain of a new penicillin (Scheme 29).[84]



(72)

Scheme 29

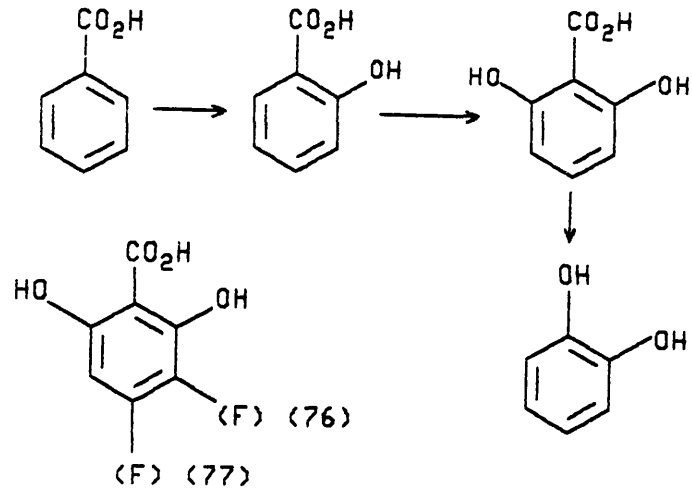
A Pseudomonas species transformed o-fluorobenzoic acid (73) into 3-fluorocatechol (74) and 2-fluoromuconic acid (75) (Scheme 30).[85]



(73) (74) (75)

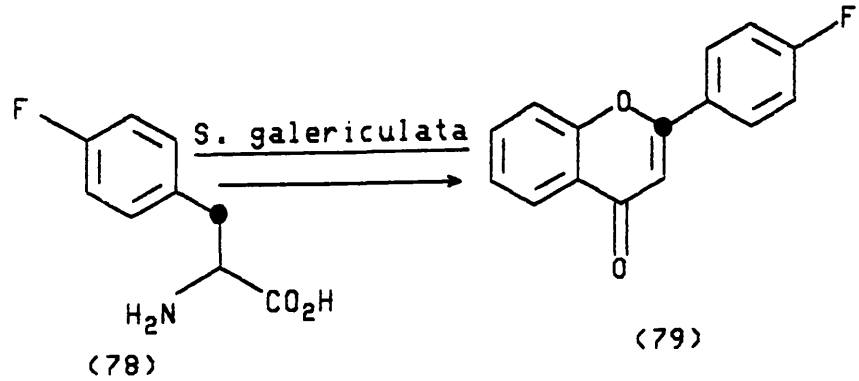
Scheme 30

Benzoic acid is converted into catechol *via* intermediate hydroxylated compounds, as shown in Scheme 31. *m*-Fluoro- and *p*-fluorobenzoic acids were also converted into the intermediate analogues (76) and (77).[86]



Scheme 31

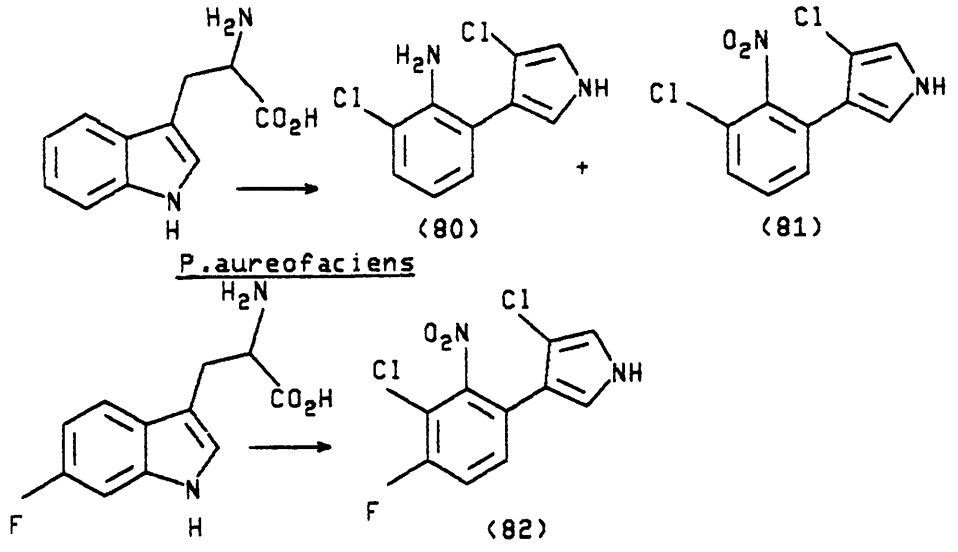
Many studies have been carried out with fluoro amino-acids and micro-organisms. [3-^{14}C]-*p*-Fluorophenylalanine (78) was converted into [2-^{14}C]-4'-fluorochrysin (79) by *Scuttellaria galericulata* (Scheme 32).[87]



(78) (79)

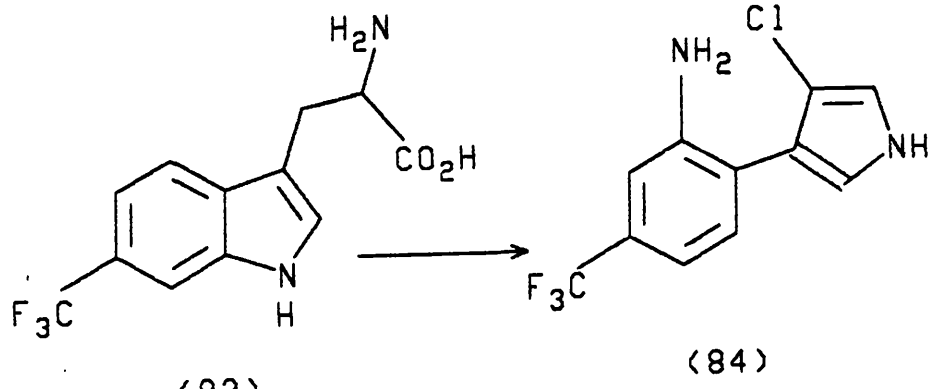
Scheme 32

Tryptophan is a precursor for the pyrrolnitrins (80) and (81) in Pseudomonas aureofaciens, and the analogue 6-fluorotryptophan was metabolised to the fluoropyrrolnitrin (82) (Scheme 33).[88]



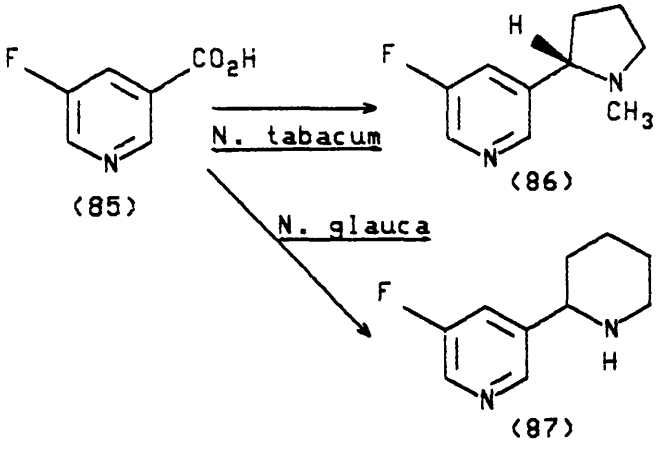
Scheme 33

In the same way, 6-trifluoromethyltryptophan (83) was converted into the analogue (84), which had not been chlorinated in the benzene ring (Scheme 34).[88]



Scheme 34

As a final example of aberrant biosynthesis using fluorine, 5-fluoronicotinic acid (85) was metabolised into the 5-fluoro analogues of nicotine (86) and anabasine (87) by Nicotiana tabacum and N. glauca respectively (Scheme 35).[89,90]



Scheme 35

In summary, analogues of natural products have been metabolised by a wide variety of biological systems. The formation of unnatural products in vivo has been useful in the preparation of analogues and the study of the metabolism and interrelationships of biologically active natural products.

The use of labelled molecules to elucidate more details of the biosynthesis of quinolizidine alkaloids is detailed in Chapter 3. The synthesis of analogues of a known precursor (cadaverine) and their effect upon plants which produce quinolizidine alkaloids is also described in Chapter 3.

CHAPTER THREE

INVESTIGATIONS INTO THE BIOSYNTHESIS OF QUINOLIZIDINE ALKALOIDS

3.1 Introduction

The majority of the biosynthetic studies upon the quinolizidine alkaloids reported to date, have been concerned with lupinine (4), and the saturated bisquinolizidines, sparteine (5) and lupanine (6). A little work has been carried out on the pyridone alkaloids N-methylcytisine (50) and cytisine (7).

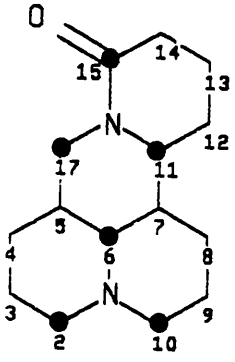
In this chapter, studies on matrine (11) and the pyridone alkaloid anagyrine (8) are detailed. Both these compounds are known to have cadaverine as a precursor and thus a series of specifically labelled cadaverines were synthesised by known routes. These precursors were fed to plants which produce the alkaloids, and the products of metabolism were analysed.

Analogues of cadaverine which contain methyl groups or a fluorine atom in place of hydrogen atoms were prepared. These compounds were fed to plants producing quinolizidine alkaloids and a study was made of their metabolism by the plants.

3.2 Biosynthesis of Matrine

A few biosynthetic investigations have been performed on matrine. Schütte and co-workers fed [1,5-^{14}C]cadaverine and [2-^{14}C]lysine to Sophora tetraptera and found that both were incor-

porated.[91] The matrine sample extracted from the plants was chemically degraded, and the labelling pattern (88) was deduced from the degradations carried out.



(88)

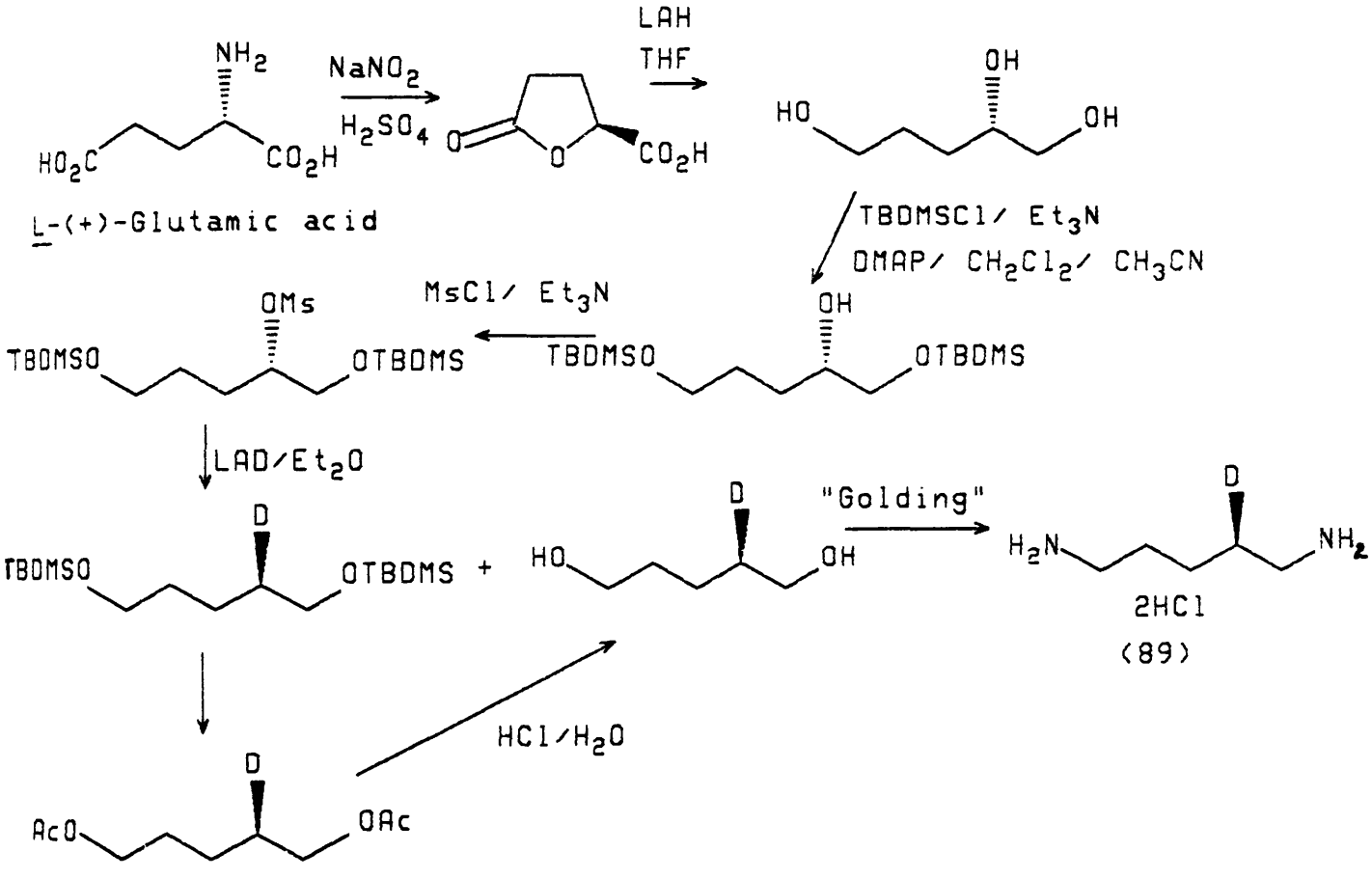
Synthetic interest in matrine has been generated as a result of its known significant antiulcer activity.[92] Matrine was first synthesised, in racemic form, by Mandell and co-workers in 1965, albeit in low yield.[93] Recently, Chen and co-workers reported a stereoselective synthesis in reasonable yield;[94] the same group also carried out a stereochemical and structural analysis of matrine using n.m.r. spectroscopy.[95]

Following on from this work, it was decided to undertake a study of the biochemical processes involved in matrine biosynthesis, by preparing labelled cadaverines and feeding them to Sophora microphylla, a matrine-producing plant. Incorporation and time study experiments on S. microphylla are discussed at the end of this chapter.

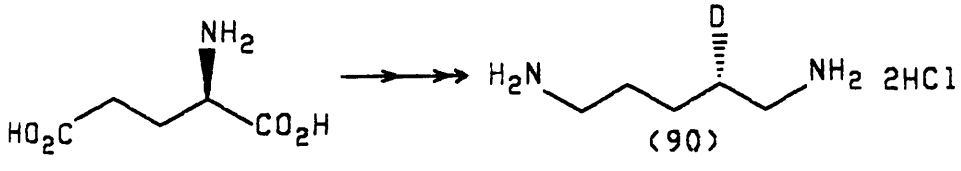
3.2.1 Synthesis of ^2H-Labelled Cadaverines

All of the cadaverines used in the experiments were synthesised by known routes.[65] The dihydrochloride of [2,2,4,4-$^2H_4$]-cadaverine (56) was prepared as shown in Scheme 23 (Chapter 2). [2,2,4,4-$^2H_4$]Glutaronitrile was prepared by exchanging the α-protons of glutaronitrile with deuterium oxide in the presence of a base, DBU. The dihydrochloride of [2,2,4,4-$^2H_4$]cadaverine (56) was formed by reduction of [2,2,4,4-$^2H_4$]glutaronitrile, followed by acidification. Inspection of the ^1H n.m.r. spectrum and the mass spectrum of this material indicated that the cadaverine (56) produced by this method contained over 92% of $^2H_4$ species. [3,3-$^2H_2$]cadaverine (57) was synthesised as the dihydrochloride salt from diethyl malonate. After exchanging the α-protons with deuterium oxide in the presence of triethylamine, the diethyl[2,2-$^2H_2$]malonate was reduced using lithium aluminium hydride. Mesylation of the diol was carried out using methanesulphonyl chloride and triethylamine followed by displacement by cyanide ion of the mesyl groups. The [3,3-$^2H_2$]glutaronitrile thus produced was reduced to the corresponding cadaverine and acidification yielded [3,3-$^2H_2$]cadaverine dihydrochloride (57) (Scheme 23). [3,3-$^2H_2$]Cadaverine dihydrochloride (57) was shown to have a ^2H content of 98% by ^1H n.m.r. and mass spectroscopy.

With a view to establishing the stereochemistry of some of the enzymic processes involved in the biosynthesis of matrine, the stereospecifically monodeuteriated (R)- and (S)-[1-^2H]cadaverines [(35) and (36) respectively] were synthesised by known routes.[48]



Similarly:-



D-(-)-Glutamic acid

Scheme 36

The enantiomeric (R)- and (S)-[1-^{2}H]cadaverine dihydrochlorides were prepared by enzymatic decarboxylation of L-lysine in ^2H_2O, and of the L-component of DL-[2-$^2H_1$]lysine in ^1H_2O, respectively, using L-lysine decarboxylase (Scheme 12, Chapter 2). The ^{2}H content of each cadaverine sample was estimated by analysing the ^{2}H n.m.r. spectrum and mass spectrum of the dihydrochlorides, and was found to be ca. 90 and 85%, respectively.

Samples of (R)- and (S)-[2-^{2}H]cadaverine dihydrochlorides (89) and (90), respectively, were kindly donated by Dr. G.N. Sheldrake, who synthesised them by the route shown in Scheme 36.

3.2.2 Feeding of ^{2}H-Labelled Cadaverines to S. microphylla

A measured quantity of each of the cadaverine dihydrochlorides was mixed with a known quantity of a radioactive tracer, [1,5-^{14}C]-cadaverine dihydrochloride. The mixture thus formed was fed to a batch of well-established Sophora microphylla plants by the wick method. Each experiment consisted of approximately 8 plants, and each precursor was administered over a period of 10-14 days. Ten to fourteen days after completion of feeding, the plants were macerated, and the total alkaloid content was isolated. The extract contained not only matrine (11), but also anagyrine (8), N-methylcytisine (50) and cytisine (7). The results from these latter alkaloids will be discussed later in this chapter. The alkaloids were separated using Kieselgel 60 F_{254} 0.25 mm thick preparative t.l.c. plates, matrine having an Rf value of 0.88. After isolation of matrine, the specific incorporations of cadaverine were determined by scintillation counting. Specific ^{14}C incorporations per

C_5 unit for a cadaverine molecule are calculated from

$$\frac{\text{Molar Activity of Product} \times \frac{1}{3}}{\text{Molar Activity of Precursor}} \times 100\%$$

Table 2 shows the ^{14}C specific incorporation per C_5 unit for each precursor.

Table 2

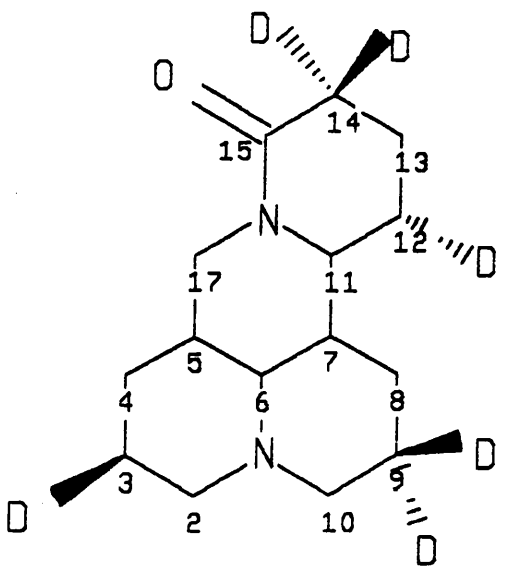
Precursor	Quantity Fed (mg)	Amount of Matrine Isolated (mg)	% ^{14}C Specific Incorporation In Matrine
(35)	200	18	2.59
(36)	200	26	3.21
(89)	325	40	2.66
(90)	215	20	2.16
(56)	200	42	2.33
(57)	120	13	4.14

In previous experiments with ^{2}H-labelled samples of quinolizidine alkaloids, the $^{2}H-\{^{1}H\}$ n.m.r. spectra in chloroform or benzene at room temperature contained very broad signals. It was discovered, however, that an increase in temperature to 60°C resulted in a narrowing of the signals: henceforth spectra were taken at this elevated temperature. The spectra quoted below were all recorded at 60°C.

A sample of matrine derived from [2,2,4,4-$^{2}H_4$]cadaverine dihydrochloride (56) displayed six peaks in the ^{2}H-{^{1}H} n.m.r. spectrum, run in benzene, at δ 2.29, 2.02, 1.47, 1.33, 1.01 and 0.87 p.p.m. (Fig. 1), corresponding to matrine with ^{2}H labels at 14β, 14α, 3β, 9β, 9α and 12α, respectively (from the published data).[95]

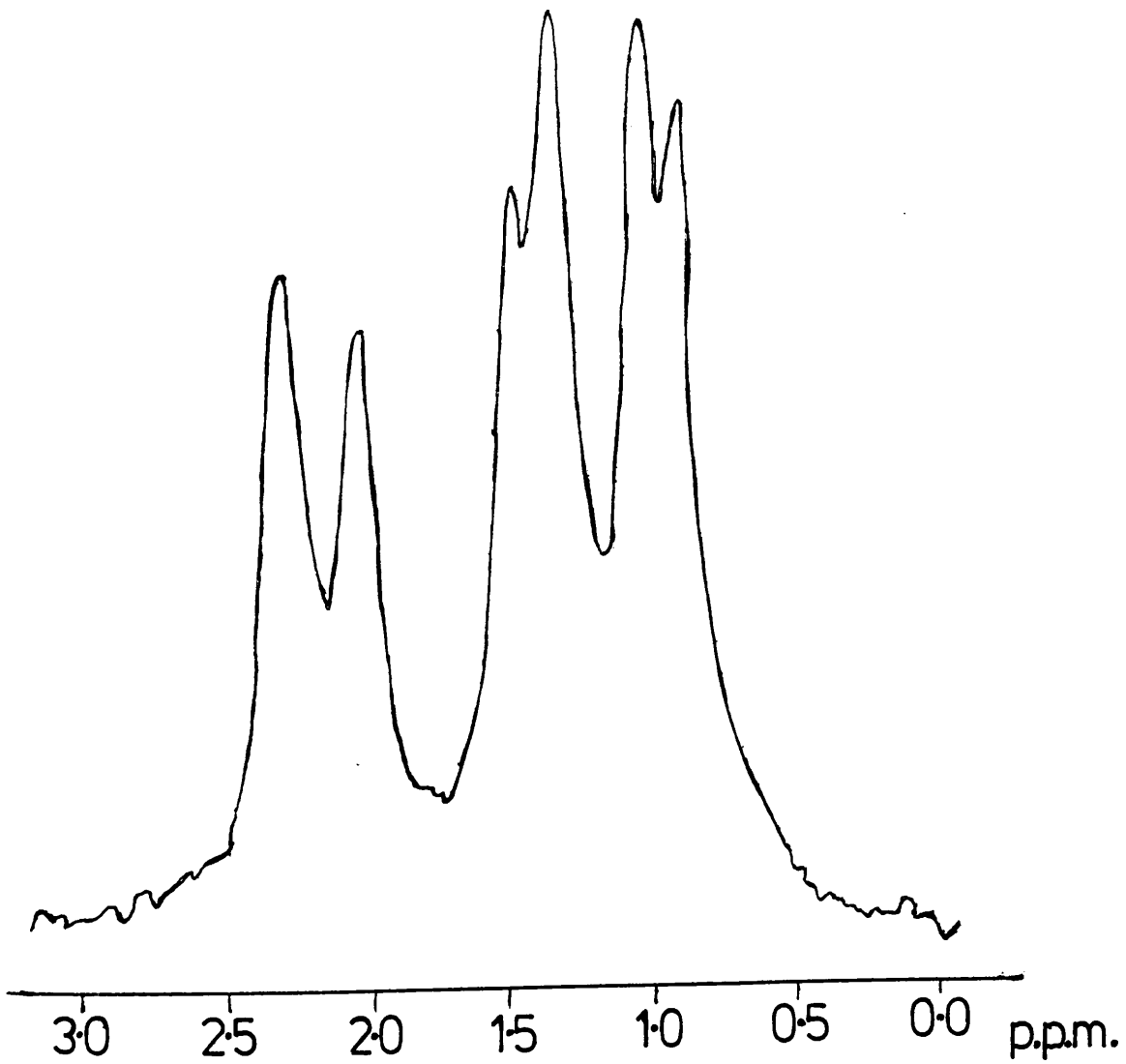
Position	^{1}H n.m.r. data (C_6D_6)	^{2}H n.m.r. data found in benzene
14β	2.37	2.29
14α	2.06	2.02
3β	1.50	1.47
9β	1.33	1.33
9α	1.03	1.01
12α	0.82	0.87

This corresponds to the labelling pattern shown in (91).



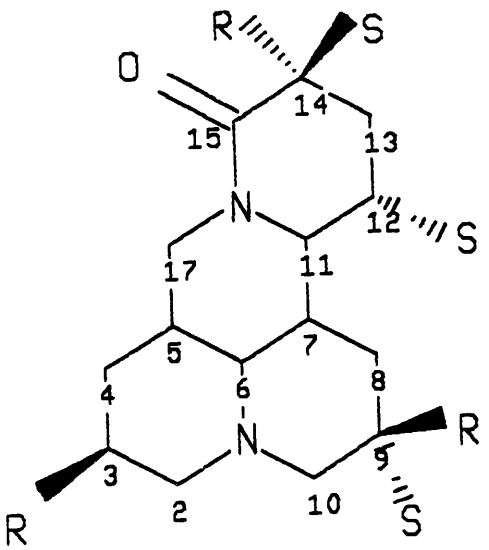
(91)

Figure 1. 55.28MHz $^{2}H-\{^{1}H\}$ N.m.r. spectrum of matrine in benzene at 60°C:



Processes are therefore occurring in the biosynthetic pathway to remove specifically the deuterium labels at 12β and 3α; and to remove the bridgehead labels at C-5 and C-7.

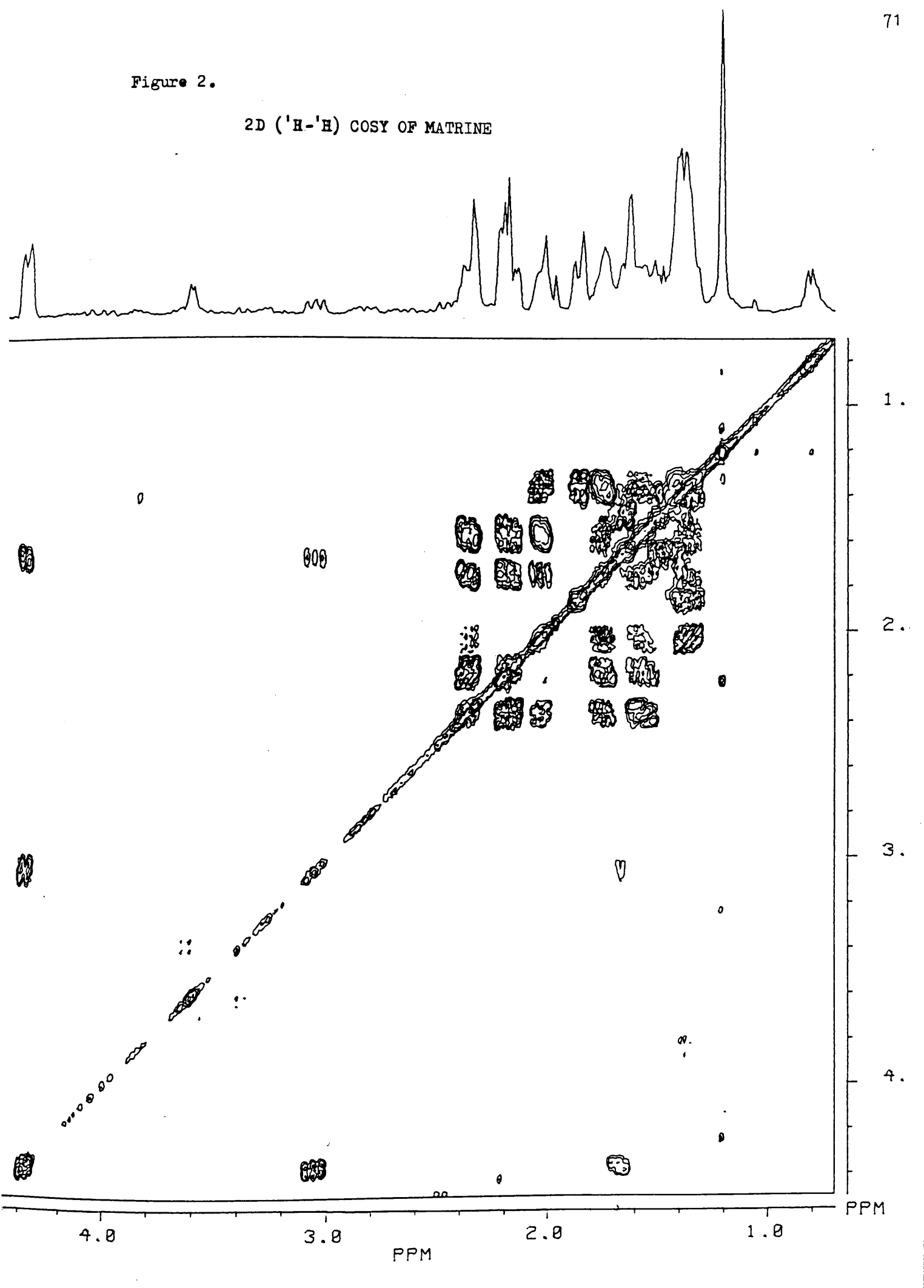
The deuterium n.m.r. spectra produced after feeding (R)- and (S)-[2-^{2}H]cadaverines complement this result - three signals appear in each spectrum. Since these spectra were run in chloroform (due to solubility problems), they must be correlated with the ^{1}H-^{1}H COSY spectrum of matrine run in chloroform (Fig. 2). Although not fully characterized, enough connections are available to locate the positions in question. The correlations indicate that the (R)-[2-^{2}H]cadaverine produced a sample of matrine with signals at δ 2.23, 2.07 and 1.64 p.p.m., corresponding to positions 14α, 9β and 3β of matrine. The (S)-[2-^{2}H]cadaverine feed gave a matrine ^{2}H n.m.r. spectrum with peaks at δ 2.57, 1.82 and 1.51 p.p.m., corresponding to positions 14β, 9α and 12α of matrine. This labelling pattern is shown in (92).



(92)

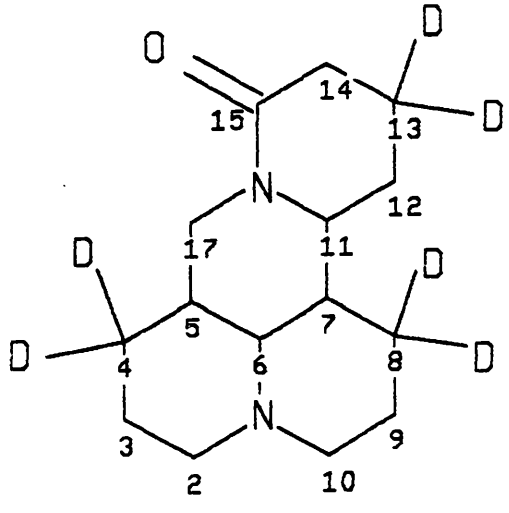
Figure 2.

2D ('H-'H) COSY OF MATRINE



The deuterium atoms are retained where no enzymic processes involving proton removal have been taking place. This leads to the appearance of ^2H at 9β and 14α from (R)-[2-^2H]cadaverine and at 9α, 12α, and 14β from the (S)-precursor. All the bridgehead deuteria are lost, invoking equilibria (enamine-imine tautomerism) which remove them. No bridgehead ^2H were observed in cytisine or N-methylcytisine after they had been fed with (R)- and (S)-[2-^2H]cadaverines in earlier biosynthetic work carried out by Robins and Sheldrake (see Chapter 2). The two deuterium atoms from C-12 and C-3 are also lost possibly due to tautomerism occurring in the biosynthetic pathway. However, the 3β atom is retained with inversion of stereochemistry. No explanation for this finding is yet available.

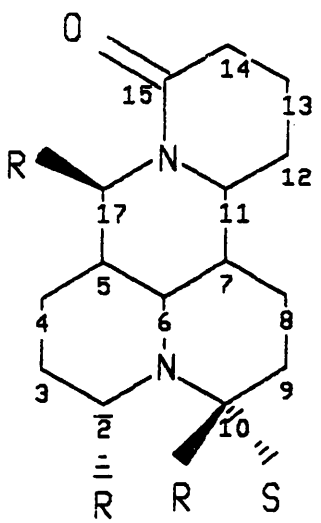
The ^2H n.m.r. spectrum of matrine obtained after feeding [3,3-$^2H_2$]cadaverine shows six peaks at δ 1.94, 1.87, 1.76, 1.75, 1.64 and 1.45 p.p.m., consistent with matrine labelled at 4β, 13α, 8β, 8α, 13β and 4α, as shown in (93).



(93)

All the deuterium atoms are retained, therefore enzymic processes involving proton removal and replacement do not occur at these positions.

The ^{2}H n.m.r. spectra of matrine resulting from the (R)- and (S)-[1-^{2}H]cadaverine dihydrochloride feeds are much less clear. Small signals in the sample derived from the (R)-precursor run in chloroform at δ 3.24, 2.80 and 2.01 p.p.m. are consistent with positions 17β, 10β and 2α of matrine being labelled (by comparison with the ^{1}H-^{1}H COSY spectrum). The ^{2}H n.m.r. spectrum of matrine from the (S)-precursor feed, which was run both in benzene and in chloroform, contains one deuterium signal, appearing at δ 1.65 p.p.m. in benzene and at δ 1.99 p.p.m. in chloroform. This peak (by comparison with published data) corresponds to position 10α of matrine. The proposed labelling pattern is shown in (94).



(94)

Due to the weak nature of the signals, the experiments were repeated in May 1989. However, specific incorporations were lower and this was reflected in poorer ^{2}H n.m.r. spectra of matrine. In order to achieve better results further studies must be carried out to obtain samples of matrine with higher enrichments of deuterium.

The data obtained establishes certain facts regarding the biosynthetic pathway to matrine. Since ^{2}H atoms appear at 10β after feeding the (R)-precursor and at 10α from the (S)-precursor, the C-N bond in cadaverine must remain intact in the formation of the N-1, C-10 bond of matrine. All the deuterium atoms which would appear at bridgeheads are lost. It is the pro-S hydrogen atoms that are removed from carbon atoms destined to become C-2 and C-17 of matrine. Both of the ^{2}H at C-2 and C-17 are retained with retention of stereochemistry. This implies that attack by a hydride donor on an intermediate N-1, C-10 iminium ion occurs from the C-si face of the intermediate and from the C-si face on an intermediate N-16, C-17 iminium ion.

3.2.3 Synthesis and Feeding of a ^{13}C-^{15}N Doubly-Labelled Cadaverine to Sophora microphylla

The labelling patterns in several quinolizidine alkaloids derived from [1-amino-^{15}N, 1-^{13}C]cadaverine (29) have been established by ^{13}C n.m.r. spectroscopy (see Chapter 2). It was decided to use this labelled cadaverine to establish the complete labelling pattern in matrine, and to show which C-N bonds from cadaverine remain intact during the biosynthetic pathway.

[1-amino-^{15}N, 1-^{13}C]Cadaverine (29) was synthesised by a known route involving treatment of 1-phthalimido-4-bromobutane with potassium [^{15}N, ^{13}C]cyanide followed by catalytic hydrogenation and removal of the protecting group by hydrolysis (Chapter 2, Scheme 7).[96] The cadaverine dihydrochloride was pulse fed with [1,5-^{14}C]cadaverine dihydrochloride to five small S. microphylla plants over four days and after a further five days the plants were harvested. The quinolizidine alkaloids were isolated in the usual way. A total incorporation of 2.7% was achieved, and 70 mg of alkaloids were extracted. The alkaloids were separated using preparative t.l.c. plates run in $CHC\ell_3$:MeOH:NH_3 (85:14:1).

In the ^{13}C-{^1H} n.m.r. spectrum of matrine taken in $CDC\ell_3$, some anagyrine was present, making the spectrum rather more complicated. On close inspection of the matrine signals, no enrichment of the ^{13}C peaks was apparent, neither were any doublets flanking natural abundance signals visible. Similarly, the ^{13}C-{^1H} n.m.r. spectrum of anagyrine showed no detectable enrichment of the ^{13}C peaks or doublets.

The poor spectra are probably due to dilution of labelled alkaloid with endogenous unlabelled material. In order to establish the labelling pattern, the experiment must be repeated, in the hope of achieving a better specific incorporation and thus obtaining more clearly defined spectra.

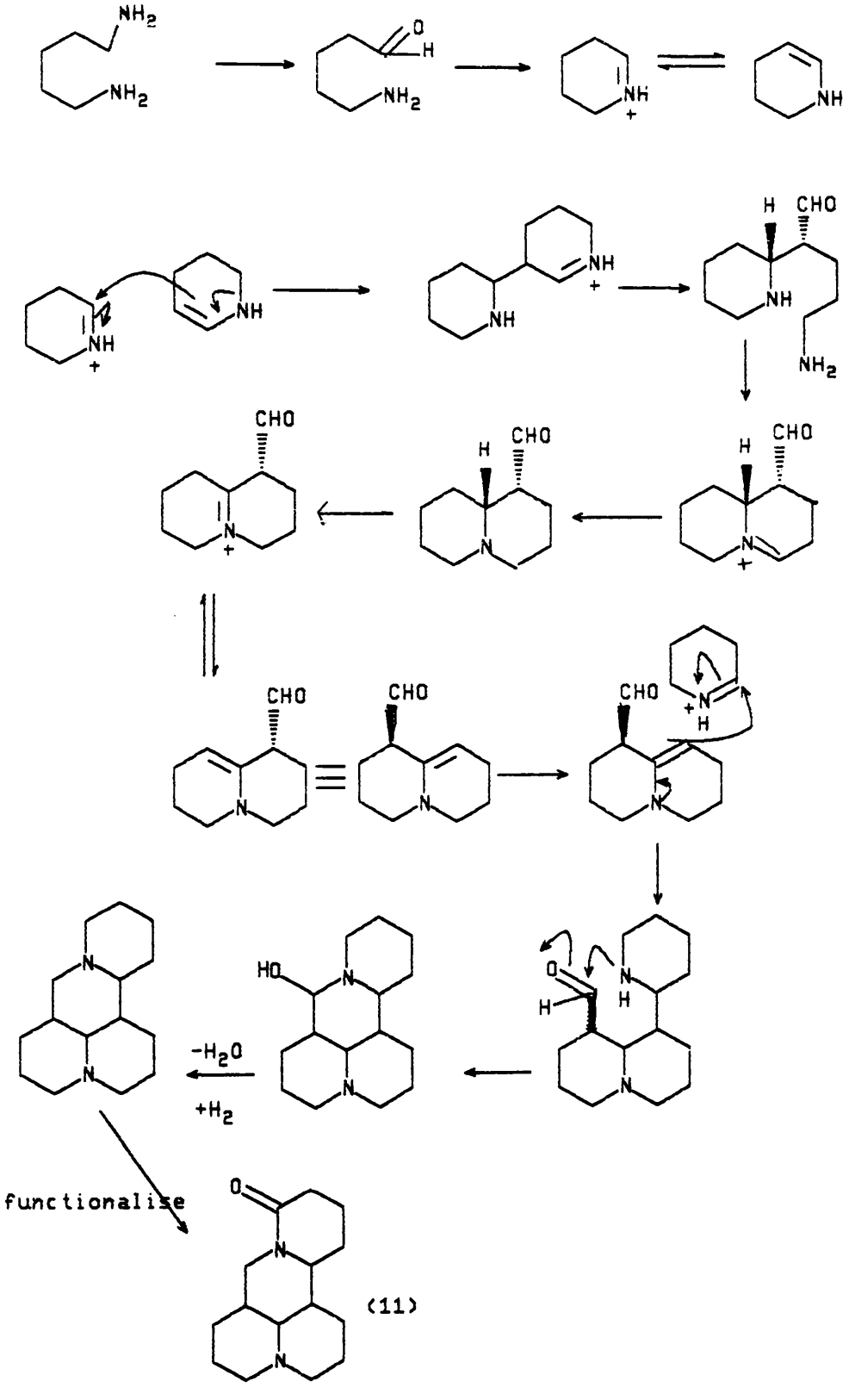
3.2.4 A Possible Biosynthesis of Matrine

A plausible biosynthesis of matrine must take into account the results of the feeding experiments with the deuterium-labelled cadaverines. Also, the postulated route should incorporate earlier results from other workers, including Spenser and Wink. Such a biosynthetic pathway is presented in Scheme 37.

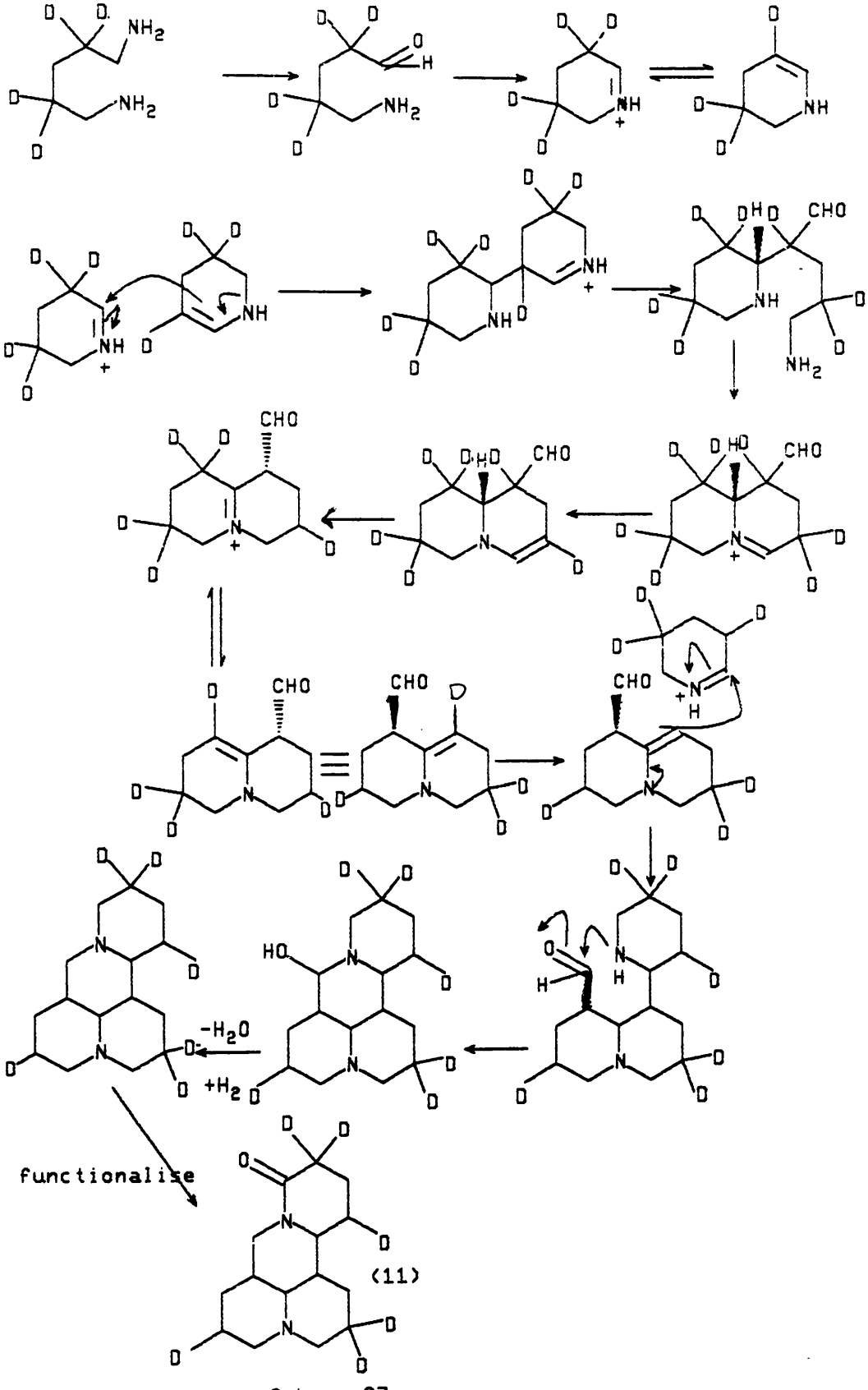
In the biosynthesis, two piperideine moieties, derived from cadaverine, couple to form tetrahydroanabasine. Tetrahydroanabasine is cleaved oxidatively and ring closure produces a lupinine-type precursor. This key intermediate combines with a third piperideine moiety to generate the matrine skeleton.

The biosynthetic pathway is illustrated for [2,2,4,4-$^2H_4$]-cadaverine in Scheme 37(a). Deuterium is lost specifically in the enamine-imine tautomerism occurring in piperideine from the position which becomes 3α in matrine. Both the deuterium atoms destined to become bridgehead hydrogens are lost, probably at the lupinine intermediate stage, where imine-enamine tautomerism can occur. Deuterium is also lost from the position which becomes 12β of matrine, presumably due to enamine-imine tautomerism. However, the inversion of stereochemistry of the two deuterium atoms retained at C-3α and C-12β is difficult to explain. The suggested pathway is consistent with the observed labelling pattern for matrine after feeding [2,2,4,4-$^2H_4$]-cadaverine.

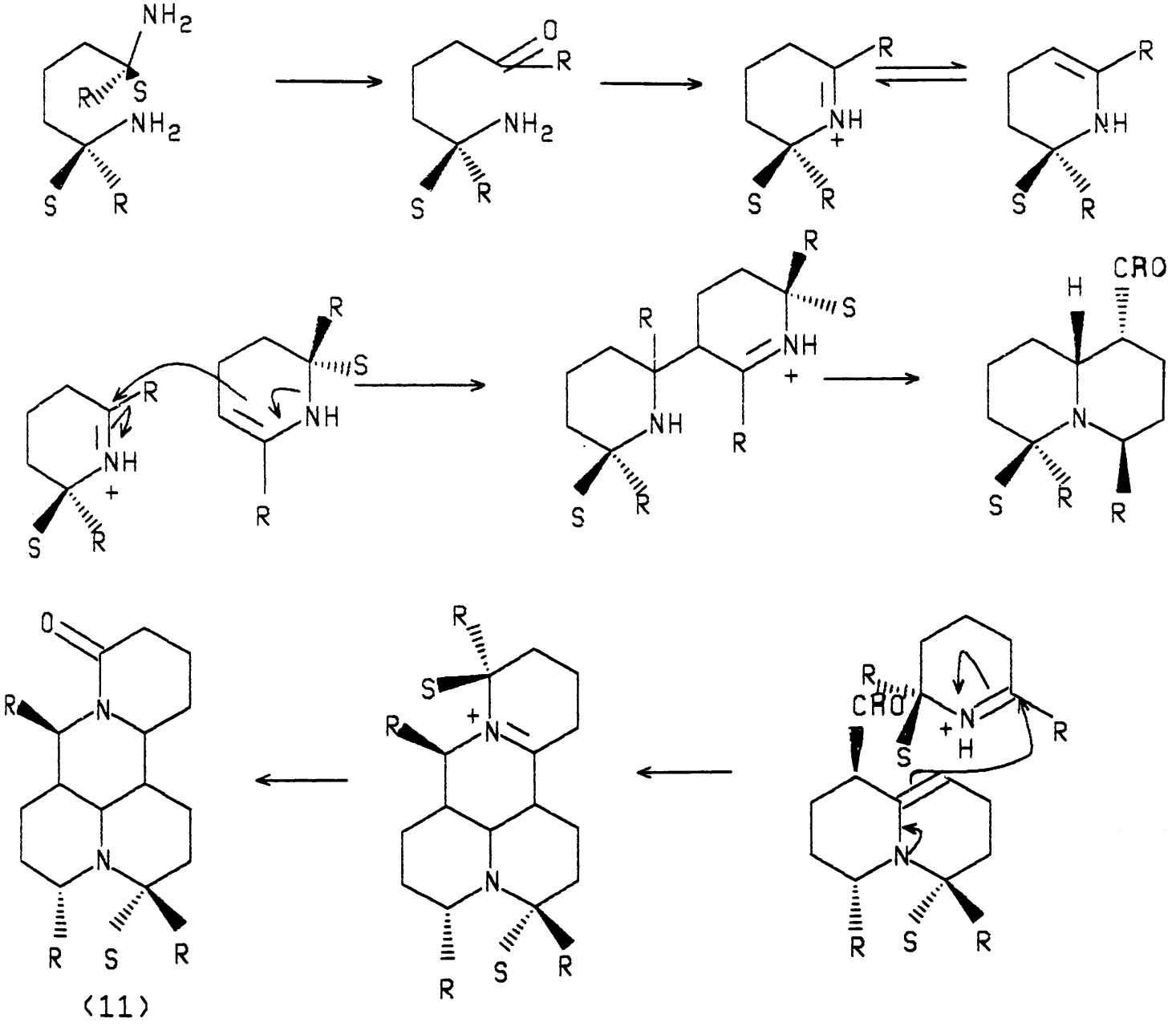
The biosynthetic pathway illustrating the fate of the (R)- and (S)-[1-^{2}H]cadaverines is shown in Scheme 37(b). It is the pro-S


NH2
NH2
O
H
NH2
NH
NH
NH
NH
NH
NH2
H
CHO
CHO
H
CHO
H
CHO
CHO
CHO
CHO
HO
O
H
-H2O
+H2
functionalise
O
(11)

Scheme 37



Scheme 37a



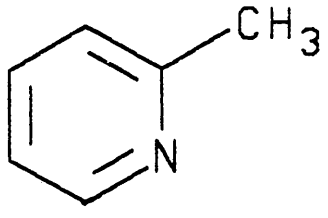
(11)

Scheme 37b

hydrogen that is lost and the pro-R hydrogen that is retained when cadaverine is oxidised. Both deuterium labels destined to become bridgehead hydrogens are lost. The deuterium atom destined to become 6α is probably lost during the oxidation-reduction processes taking place at the lupinine intermediate stage since one proposed intermediate has a double bond at C-6,7 of matrine. The deuterium label at C-11 of matrine must be lost towards the end of the biosynthetic pathway - loss of bridgehead atoms is not uncommon in the biosynthesis of quinolizidine alkaloids. The proposed pathway is, therefore, consistent with the labelling patterns observed after feeding (R)- and (S)-[1-^{2}H]cadaverines.

3.3 The Biosynthesis of Anagyrine

Anagyrine (8) has been found in several species of Lupinus and Genista and has been isolated from gorse. Its absolute configuration was determined by Okudo by relating anagyrine to (+)-epilupinine.[97] An early synthesis of anagyrine was achieved from α-picoline (95) by Van Tamelen and Baran.[98]



(95)

No biosynthetic experiments have been carried out on anagyrine. It was, therefore, felt necessary to undertake some studies of the biosynthesis of anagyrine. The alkaloid biosynthesis was studied in

Anagyris foetida, where it is the major product (N-methylcytisine is the other alkaloid produced).

Samples of (R)- and (S)-[1-^{2}H]-, (R)- and (S)-[2-^{2}H]-cadaverines were prepared as dihydrochlorides as previously described. Each precursor was fed alongside a ^{14}C-labelled cadaverine to eight A. foetida plants by the wick method of feeding. Feeding was carried out over a period of 12 days, then the plants were left for a further 12 days before they were harvested. The alkaloidal mixture was extracted in the usual way, and separation of the alkaloids was accomplished using preparative t.l.c. plates. The plates were developed in chloroform/methanol/conc. ammonia (85:14:1) and the scrapings were eluted with methanol and a few drops of ammonia. Incorporation levels for the precursors into the alkaloids were determined by scintillation counting. Table 3 details the ^{14}C specific incorporation per C_5 unit for each precursor.

Table 3

Precursor	Quantity Fed (mg)	Amount of Anagyrine (mg)	% ^{14}C Specific Incorporation per C_5 unit
(35)	191	368	0.79
(36)	211	230	1.39
(89)	217	112	1.18
(90)	198	326	1.00

Table 4

Assignments for Anagyrine

^{1}H n.m.r. spectrum, $CDC\ell_3$, 298K, ref. $CHC\ell_3$ at δ 7.25 p.p.m.

δ/p.p.m.	Position
0.90	14β
0.92	12β
ca. 1.23	13β
ca. 1.35	14α
1.43	8 pro-R
ca. 1.65	12α
ca. 1.65	13α
1.77	8 pro-S
1.91	9
2.23	17β
ca. 2.39	15α
ca. 2.48	15β
2.65	11
2.74	7
3.15	17α
3.64	10α
3.82	10β
5.75	5
6.17	3
7.03	4

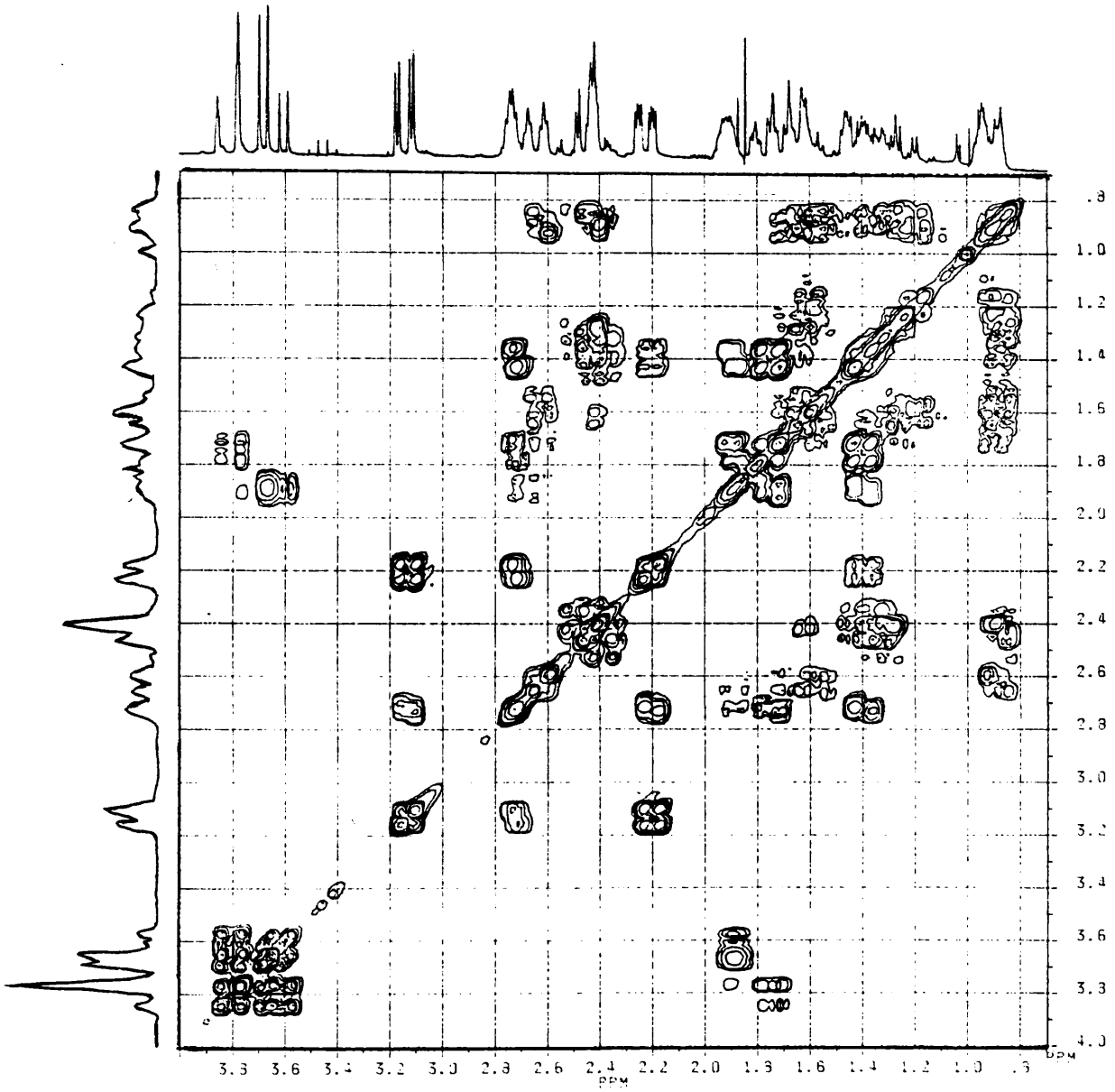
Table 5

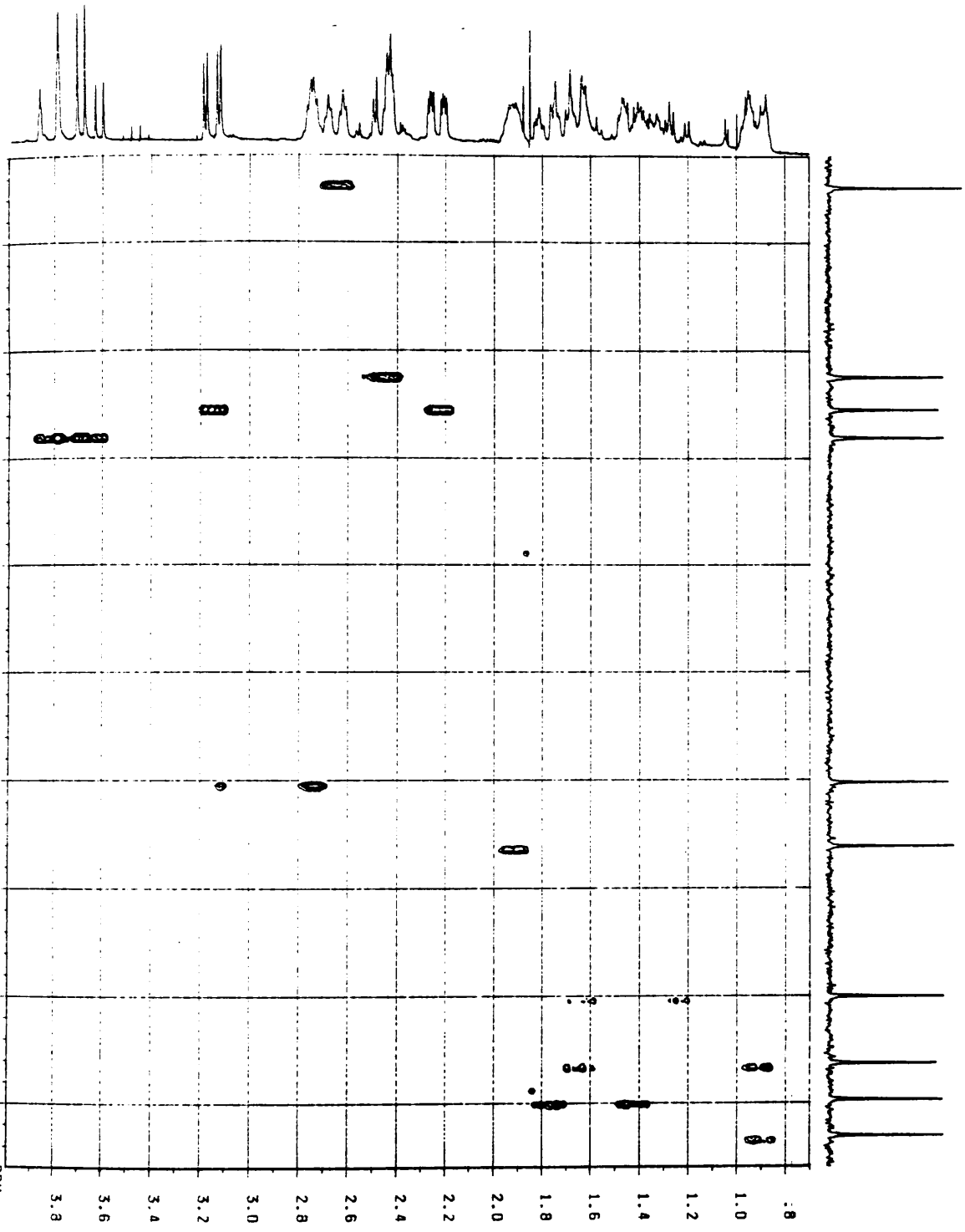
Assignment of Signals for Anagyrine

^{13}C n.m.r., 298K, ref. $CDC\ell_3$ at δ 77.0 p.p.m.

δ/p.p.m.	Carbon Atom
18.5	14
20.2	8
21.9	12
25.0	13
32.0	9
34.9	7
51.0	10
52.3	17
53.8	15
62.6	11
104.0	5
115.8	3
138.2	4
151.6	6
162.9	2

2D (^{1}H-^{1}H) COSY OF ANAGYRINE.

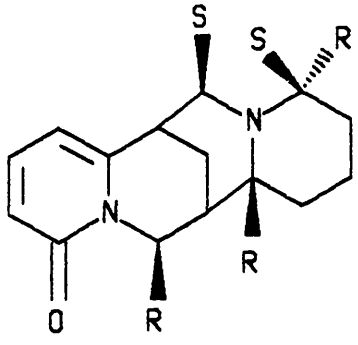




2D (^{13}C-^{1}H) COSY OF ANAGYRINE.

Since no proton assignments were available for anagyrine, it was necessary to perform some n.m.r. 2D correlation studies. These studies were carried out by Dr. D. Rycroft of the Chemistry Department at Glasgow University. Tables 4 and 5 detail the provisional assignments - studies are still in progress, in order to confirm the assignments.

The anagyrine derived from (R)-[1-^{2}H]cadaverine contained three peaks in the deuterium n.m.r. spectrum, at δ 4.02, 2.79 and 2.63 p.p.m., corresponding to anagyrine with deuterium labels at 10β, 11 and 15α (Fig. 3). The ^{2}H n.m.r. spectrum of anagyrine produced from (S)-[1-^{2}H]cadaverine had signals at δ 2.67 and 2.41 p.p.m., corresponding to positions 15β and 17β (Fig. 4). The composite labelling pattern for anagyrine after feeding (R)- and (S)-[1-^{2}H]-cadaverines is shown below in (96).



(96)

Earlier feeding experiments with (R)- and (S)-[1-^{2}H]-cadaverines to Baptisia australis produced the labelling patterns (54) and (55) for (+)-sparteine and (-)-N-methylcytisine.

Figure 3 and Figure 4. 55.28 MHz ^2H-{^1H} N.m.r. spectrum of anagyrine in chloroform at 60°C.

(a) sample of anagyrine derived from (R)-[1-^2H]-cadaverine;

(b) sample of anagyrine derived from (S)-[1-^2H] cadaverine.

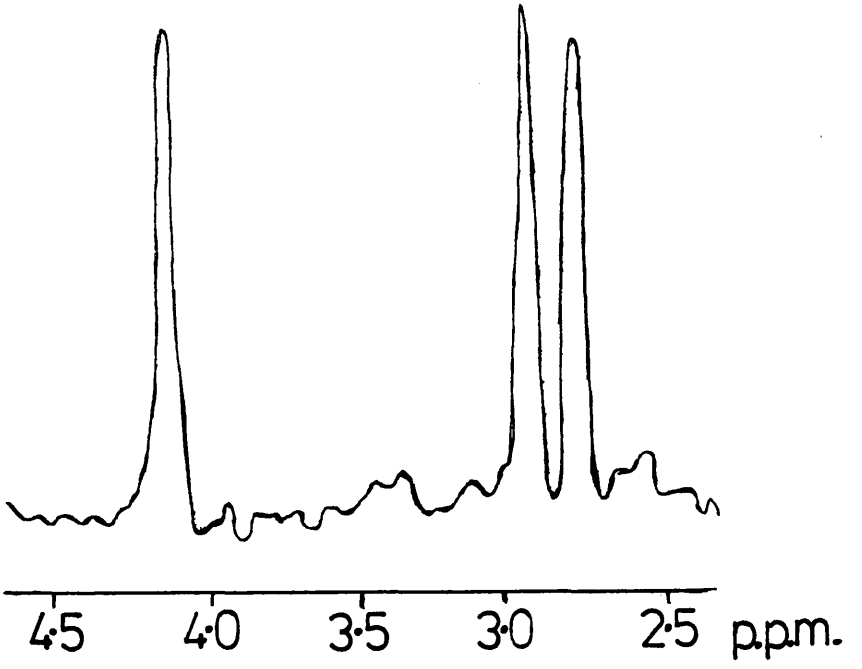


Figure 3

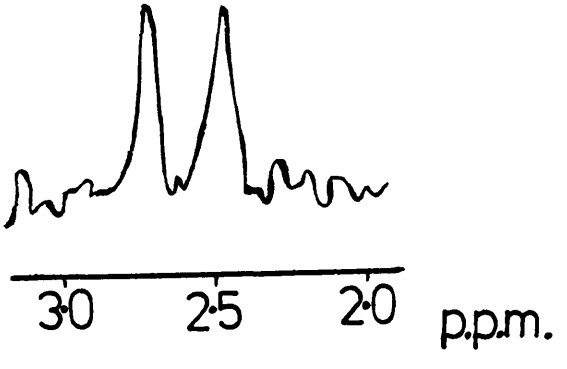
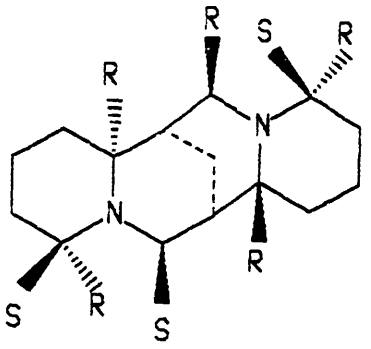
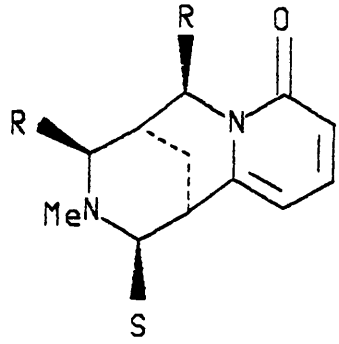


Figure 4



(54)

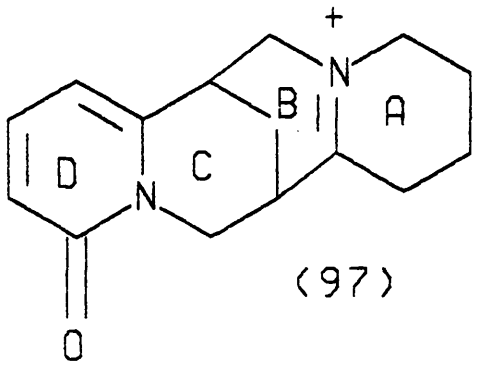


(55)

As stated in Chapter 2, if the two molecules are derived from the same tetracyclic precursor, then ring A must be cleaved and ring D oxidised to the pyridone to produce the observed signals at C-10 and C-13 of N-methylcytisine. The ^{2}H label of C-11 of N-methylcytisine, derived from (R)-[1-^{2}H]cadaverine is retained on cleavage of ring A, but its stereochemistry is inverted. This can be explained by invoking a C-11, N-12 immonium ion intermediate which is reduced stereospecifically from the re-face.

When the anagyrine labelling pattern is compared with those of (+)-sparteine and N-methylcytisine, it is clear that ring D must be converted to the pyridone ring if anagyrine is derived from the same tetracyclic precursor. However, in that case, the configuration of the proton on C-6 of anagyrine is opposite to that in sparteine. This feature is difficult to explain, since no iso-sparteine (with C-6β configuration) has ever been isolated in A. foetida, only (+)-sparteine. A possible explanation is that one of the steps in the biological pathway to the two alkaloids, anagyrine and N-methylcytisine, involves an immonium

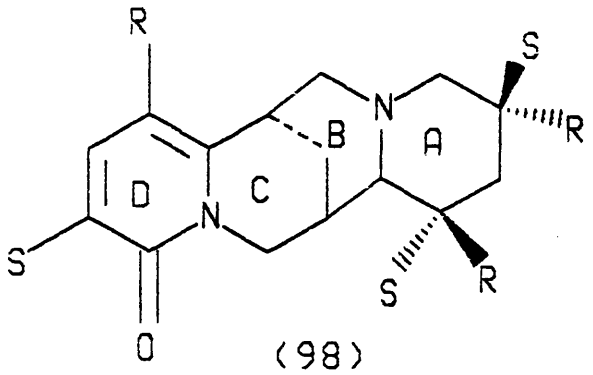
ion intermediate (97), held on the enzyme surface.



(97)

This intermediate could be reprotonated from the re-face to give anagyrine with the correct stereochemistry, or could be oxidised followed by cleavage of ring A to produce N-methylcytisine. It is highly unlikely, however, that an enzyme would reprotonate (97) with the deuterium atom abstracted. Integration of the peaks in the ^{2}H n.m.r. shows that this signal is of similar enrichment to the other peaks. More work is necessary if the pathway is to be elucidated further.

The signals in the spectra of anagyrine derived from (R)- and (S)-[2-^{2}H]cadaverine were very weak. The natural abundance peaks could be observed in the spectra. The spectrum from anagyrine fed with (R)-[2-^{2}H]cadaverine showed three small peaks at δ 5.97, 1.64 and 1.20 p.p.m., corresponding to positions 5, 12β and 14α. The ^{2}H n.m.r. spectrum of anagyrine derived from the (S)-precursor contained signals at δ 6.41, 1.85 and 1.16 p.p.m., corresponding to deuterium labels at positions 3, 12α and 14β. The composite labelling pattern is shown below (98).



(98)

It is the pro-R proton that is retained at the carbon atom which becomes C-5 in anagyrine, and it is the pro-S proton that is retained at the C-3 position in the formation of the pyridone ring.

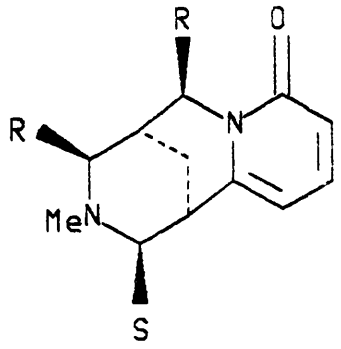
The stereochemistry of various enzymic processes in the biosynthesis of anagyrine have now been established, but more work is required to establish the complete biological pathway to anagyrine.

3.4 Biosynthesis of Cytisine and N-Methylcytisine

Information was gained regarding the biosynthetic pathway to cytisine (7) and N-methylcytisine (50) while studying the quinolizidine alkaloids matrine (11) and anagyrine (8) in Sophora microphylla and Anagyris foetida.

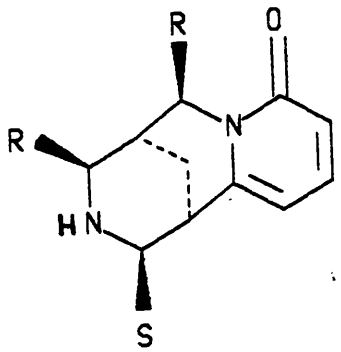
After feeding (R)- and (S)-[1-^{2}H]cadaverine dihydrochlorides [(35) and (36) respectively] to S. microphylla, the alkaloidal mixture was extracted and separated using preparative t.l.c. plates. Cytisine and N-methylcytisine were isolated along with matrine and anagyrine. Similarly, feeding the cadaverine precursors to A. foetida plants gave labelling patterns not only for anagyrine but also for N-methylcytisine.

The ^{2}H n.m.r. spectrum of N-methylcytisine, run in chloroform, derived from (R)-[1-^{2}H]cadaverine showed two deuterium signals at δ 4.01 and 2.83 p.p.m. corresponding to N-methylcytisine with deuterium labels at positions 10β and 11β. The deuterium spectrum of N-methylcytisine obtained after feeding the (S)-precursor contained one peak at δ 2.79 p.p.m. corresponding to position 13β. The composite labelling pattern obtained is shown in (55), confirming the labelling pattern obtained from previous experiments.



(55)

The ^{2}H n.m.r. spectrum of cytisine obtained after feeding (R)-[1-^{2}H]cadaverine to S. microphylla contained two peaks at δ 4.11 and 3.10 p.p.m., corresponding to cytisine with deuterium labels at positions 10β and 11β. The spectrum produced after feeding the (S)-precursor shows a signal at δ 3.00 p.p.m., corresponding to position 13β. The composite labelling pattern is shown in (99).



(99)

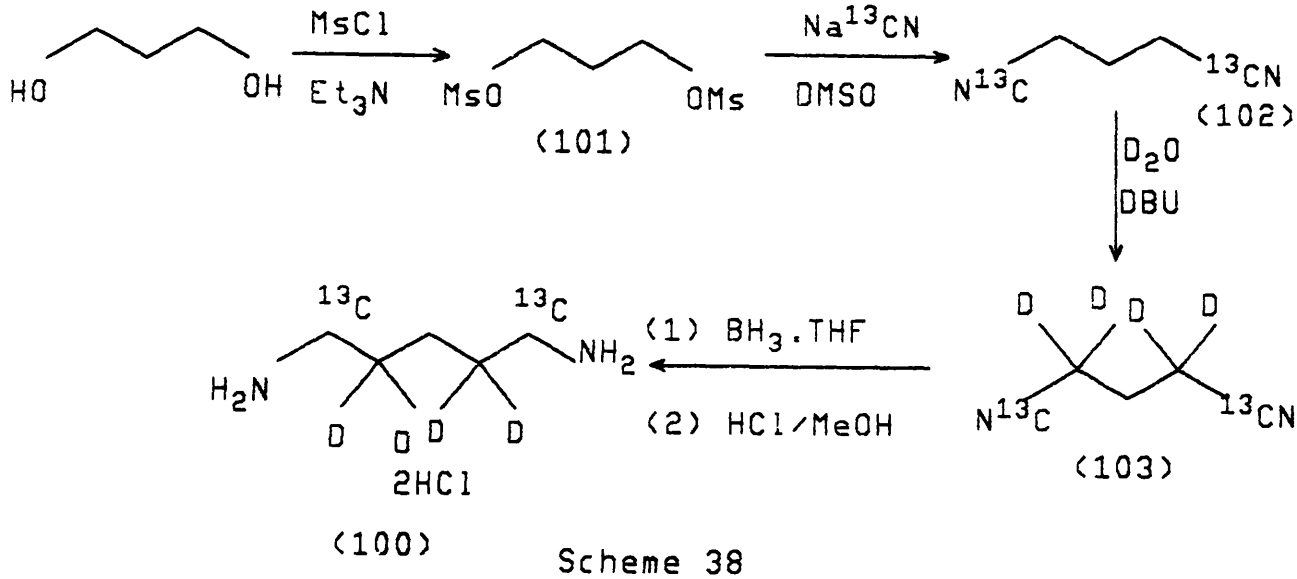
Again, these results are in agreement with those obtained from previous experiments.

3.5 The Biosynthesis of Lupinine and Sparteine

Many of the investigations into the biosynthesis of lupinine (4) and sparteine (5) have already been discussed in Chapter Two. Difficulties in the interpretation of the ^{2}H n.m.r. spectrum of lupinine arose after Robins and Sheldrake fed (R)- and (S)-[2-^{2}H]cadaverine dihydrochlorides (89) and (90) to L. luteus plants. The positions corresponding to the ^{2}H signals could not be determined. An experiment was devised, therefore, to ascertain which deuterium atoms were retained in the production of lupinine. This was carried out by preparing the precursor [1,5-$^{13}C_2$; 2,2,4,4-$^{2}H_4$]cadaverine dihydrochloride (100), in which the ^{13}C-enriched positions are beta to deuterium atoms, and will be shifted in the ^{13}C n.m.r. spectrum of the isolated lupinine, if ^{2}H is retained adjacent to the ^{13}C.

The cadaverine precursor was synthesised as shown in Scheme 38.

Propane-1,3-diol was treated with triethylamine and methanesulphonyl chloride to afford propane-1,3-diol dimethanesulphonate (101) in 72% yield. Displacement of the methanesulphonate group of (101) by sodium[^{13}C]cyanide was achieved in 63% yield to produce [1,5-$^{13}C_2$]-glutaronitrile (102). Exchange of the protons by deuterium was accomplished using 2.2 molar equivalents of DBU. A 38% chemical yield and a 90% deuterium exchange were achieved. Employing less molar

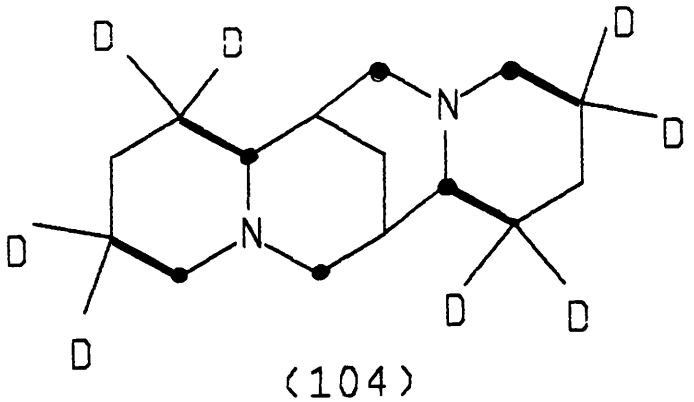


Scheme 38

equivalents had resulted in higher chemical yields, but unacceptable deuterium exchanges - 0.53 equivalents of DBU afforded the product in 64% chemical yield, 78% deuterium exchange, while one equivalent of DBU resulted in 50% chemical yield and 85% deuterium exchange in the product. Diborane reduction of [1,5-$^{13}C_2$]glutaronitrile (103), gave the final product (100) in 37% yield.

The cadaverine precursor (100) was fed to 8 L. luteus plants with a spike of ^{14}C-labelled cadaverine (1.6 μCi) using the xylem pricking method. Administration of the compound in this way involves placing droplets of solution at nodes or on the stem of the plant, and piercing the plant through the droplet. The solution is then taken up by the plant through the xylem vessels. The plants were fed over a

period of one week, and were harvested after a further period of two weeks. The alkaloidal material was extracted, and a specific incorporation of 7.7% was achieved. The crude alkaloidal mixture was separated on a basic alumina column to yield lupinine (4) and sparteine (5). The ^{13}C n.m.r. spectrum obtained for sparteine is shown in Fig. 5, and Fig. 6 illustrates an expansion of the lower field peaks. The peaks at 66.45, 66.36, 56.44 and 55.70 p.p.m. have two upfield doublets, corresponding to carbon atoms 11, 6, 2 and 15, while the signals at 66.20 and 55.81 p.p.m. appear as singlets, corresponding to carbon atoms 10 and 17. The sparteine spectrum was recorded as its dihydrochloride. The labelling pattern is shown in (104).



(104)

The observed doublets correspond to a β-isotopic shift for ^{13}C atoms with one deuterium and with two deuteriums beta, with the splitting being due to ^{13}C-^{13}C coupling through the nitrogen atom e.g. C-2 with C-6 etc. The biosynthetic pathway involves loss of the bridgehead deuterium labels, as observed in anagyrine, N-methylcytisine and cytisine.

Figure 5. 50 MHz ^{13}C-{^{1}H} N.m.r. spectrum of sparteine in benzene.

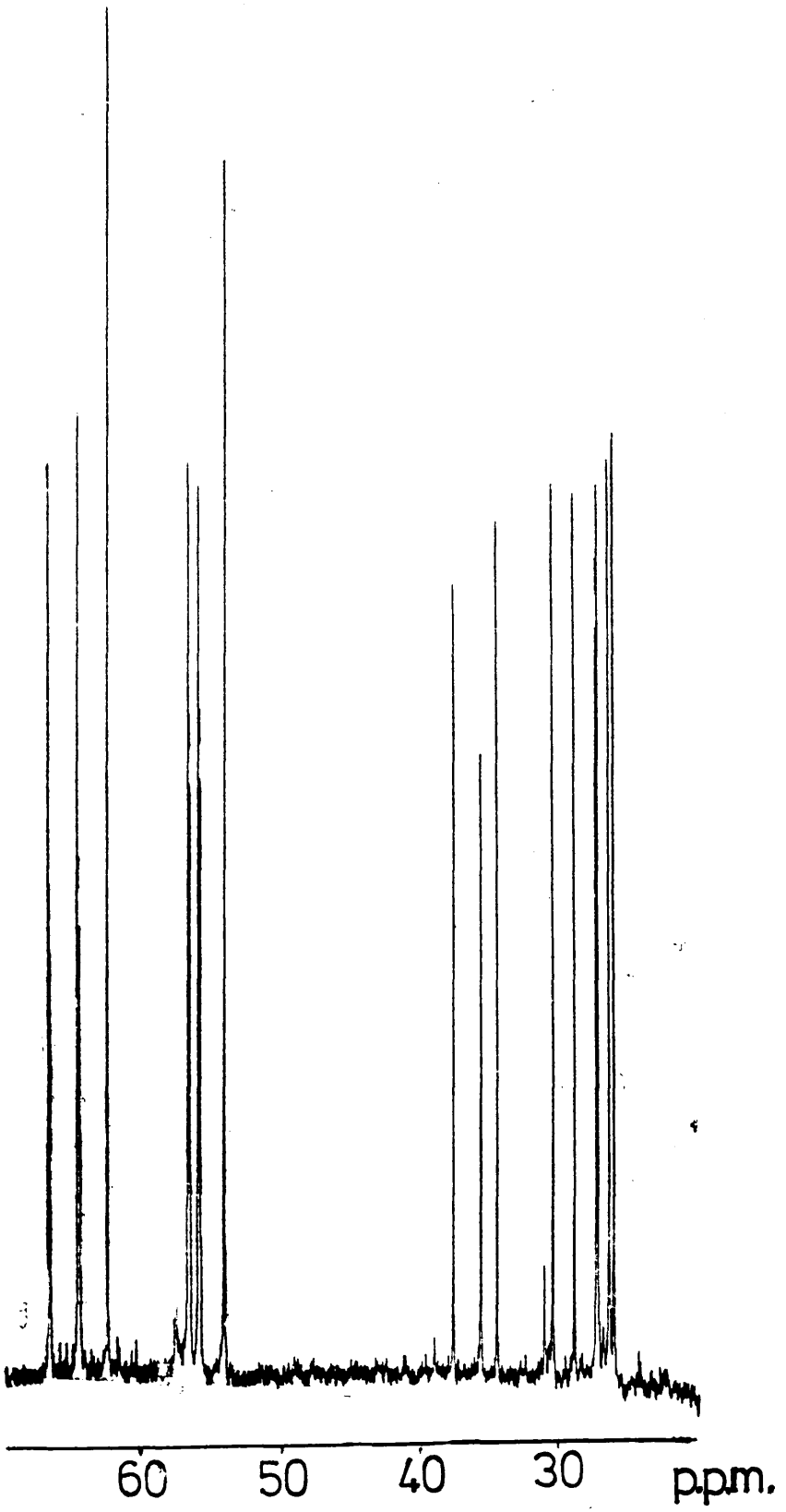
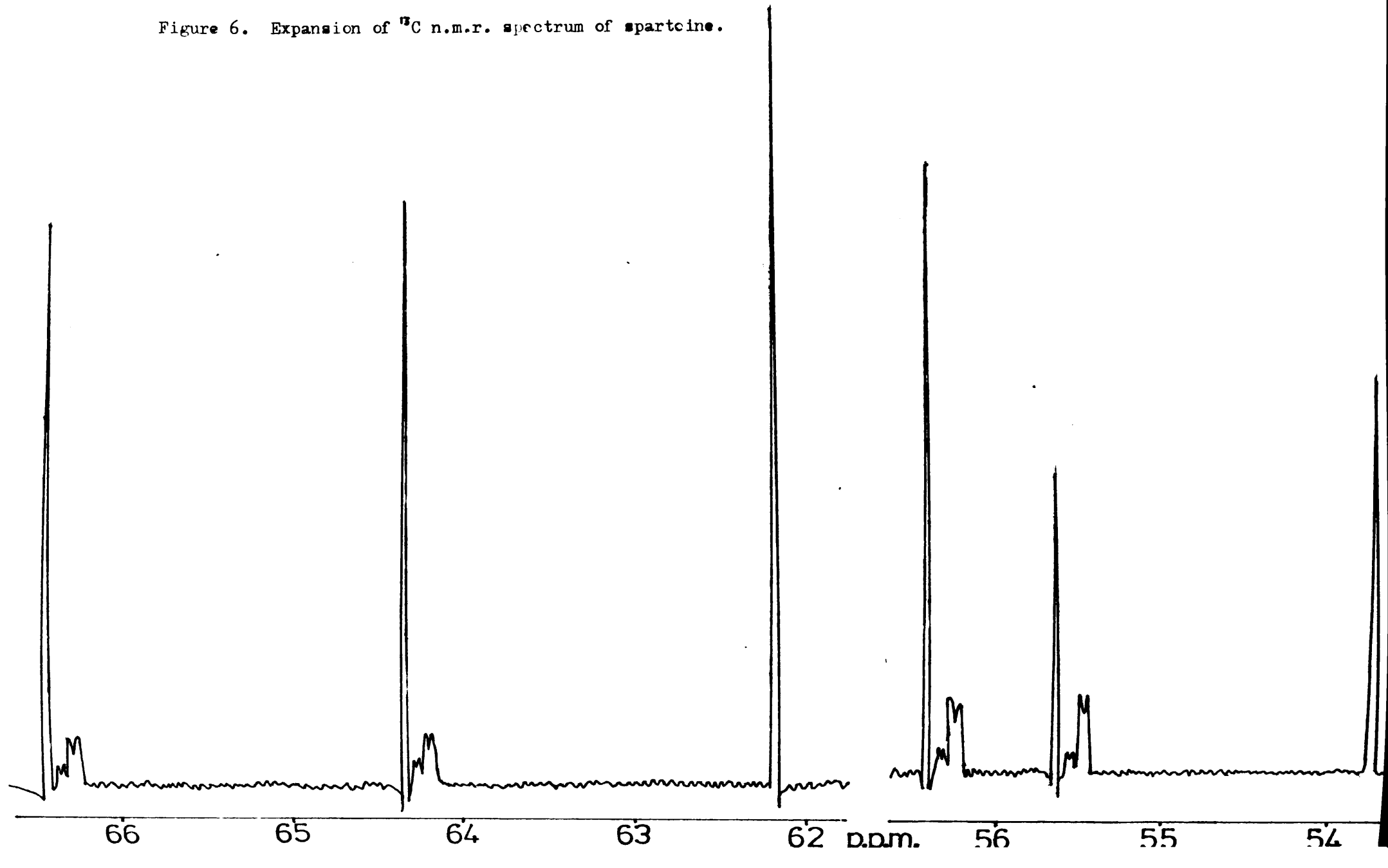
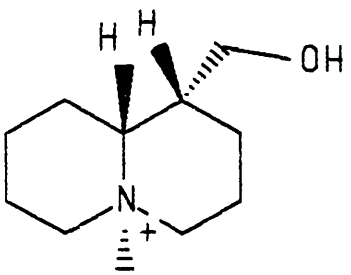


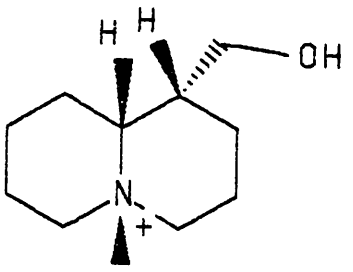
Figure 6. Expansion of ^{13}C n.m.r. spectrum of sparteine.



The lupinine example is a more complicated one. The ^{13}C n.m.r. spectrum of lupinine hydrochloride (Fig. 7) consists of a mixture of the cis and trans isomers (106) and (105), respectively.



(105)



(106)

There are four signals which appear as doublets, corresponding to C-10 and C-6 in both the cis and trans forms. The peaks corresponding to carbons 4 and 11 do not appear as doublets. The C-11 signals are sharp singlets, indicating there are no deuterium atoms beta to the carbon atom, while the C-4 peaks are broad signals, perhaps showing the presence of a single deuterium. The presence of a single deuterium would be consistent with the biosynthetic pathway proposed for matrine (11), discussed earlier. In order to clarify this spectrum, the salt was basified and the spectrum of free lupinine was recorded. This is shown in Fig. 7(a), and the expansions in Fig. 8. Carbon atom C-11 appears as a singlet and thus confirms that there is no deuterium present at the C-1 position of lupinine. This is a similar pattern to that of sparteine for the C-17 position, although the spectrum is not so well defined. Furthermore, the labelling pattern in lupinine is consistent with there

Figure 7. 50 MHz $^{13}C-\{^{1}H\}$ N.m.r. spectrum of lupinine hydrochloride in chloroform.

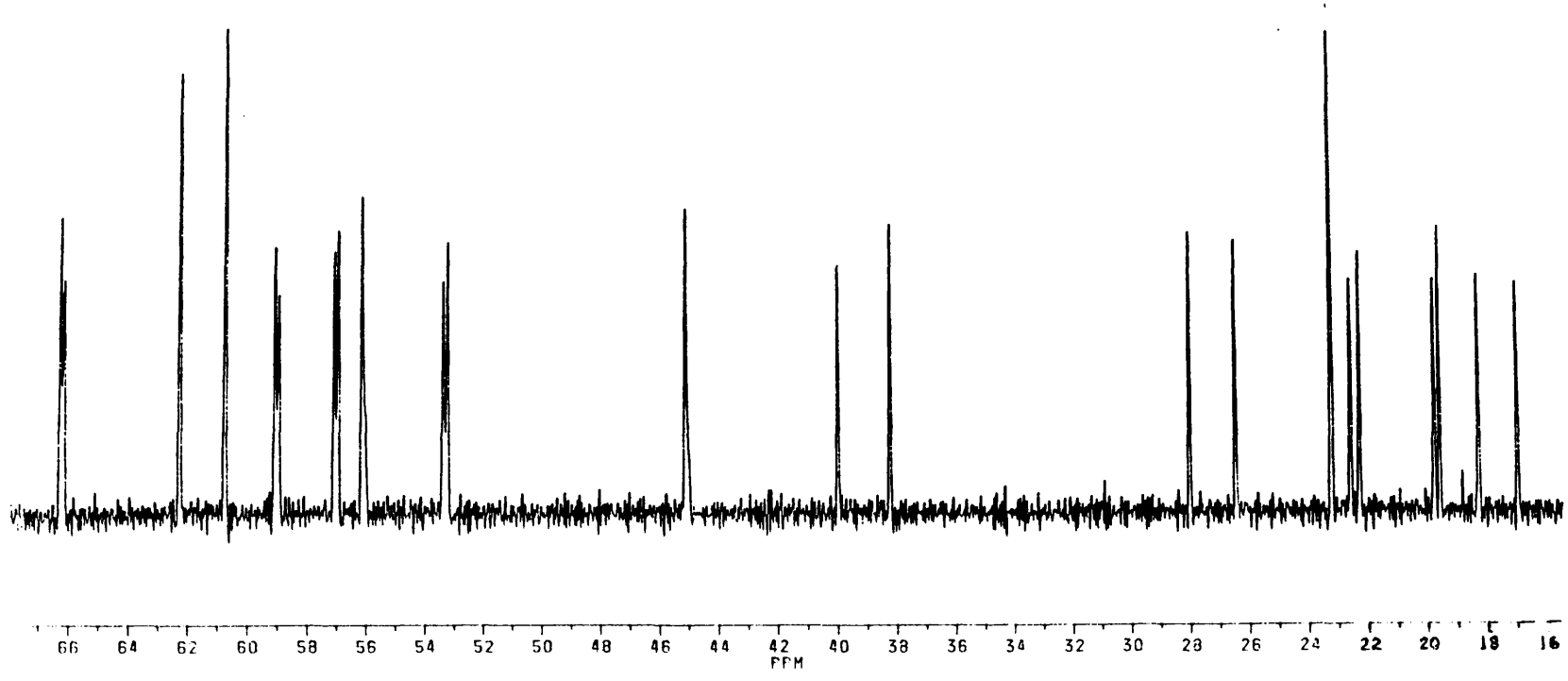


Figure 7(a). 50 MHz $^{13}C-\{^{1}H\}$ N.m.r. spectrum of lupinine in chloroform.

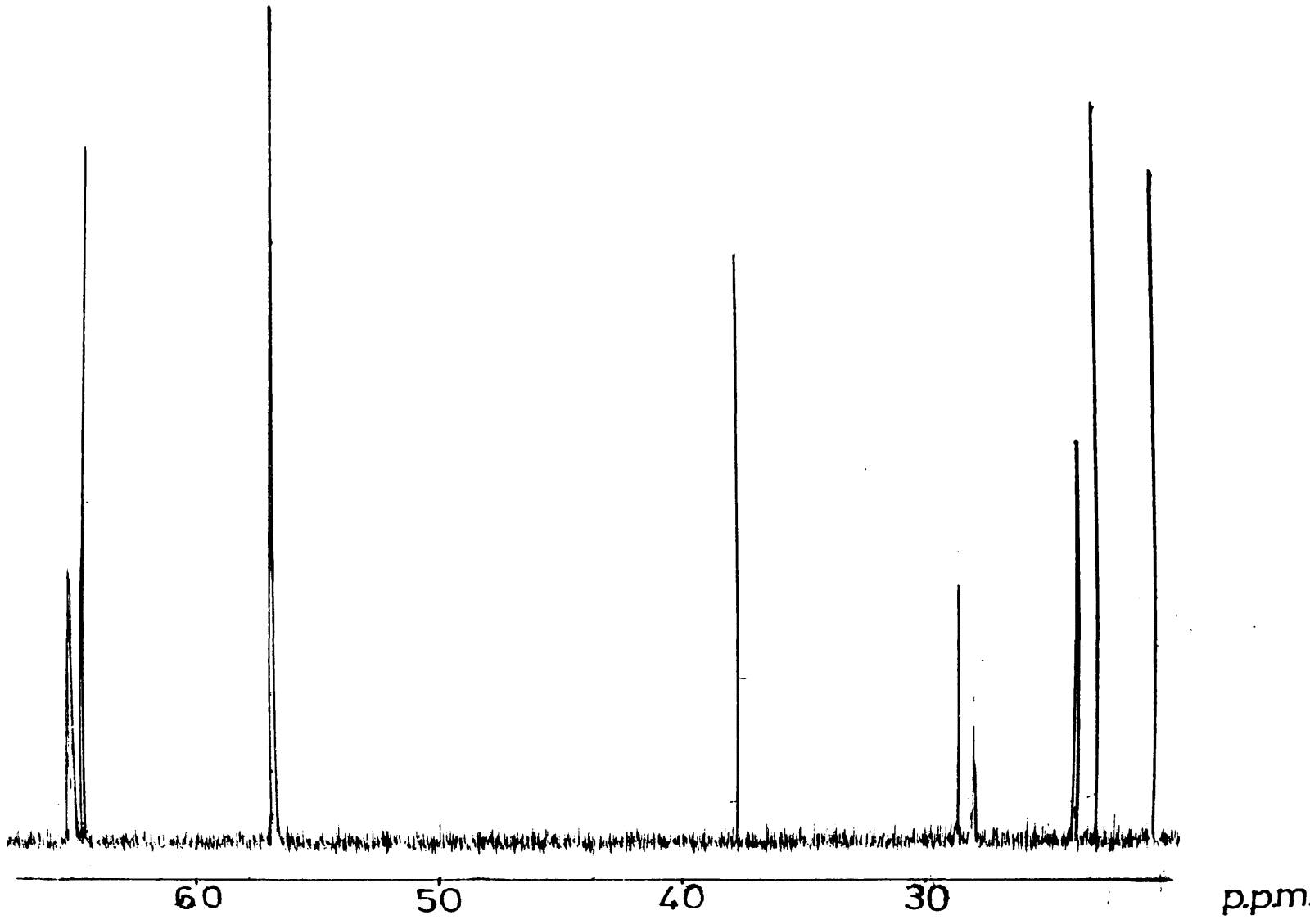
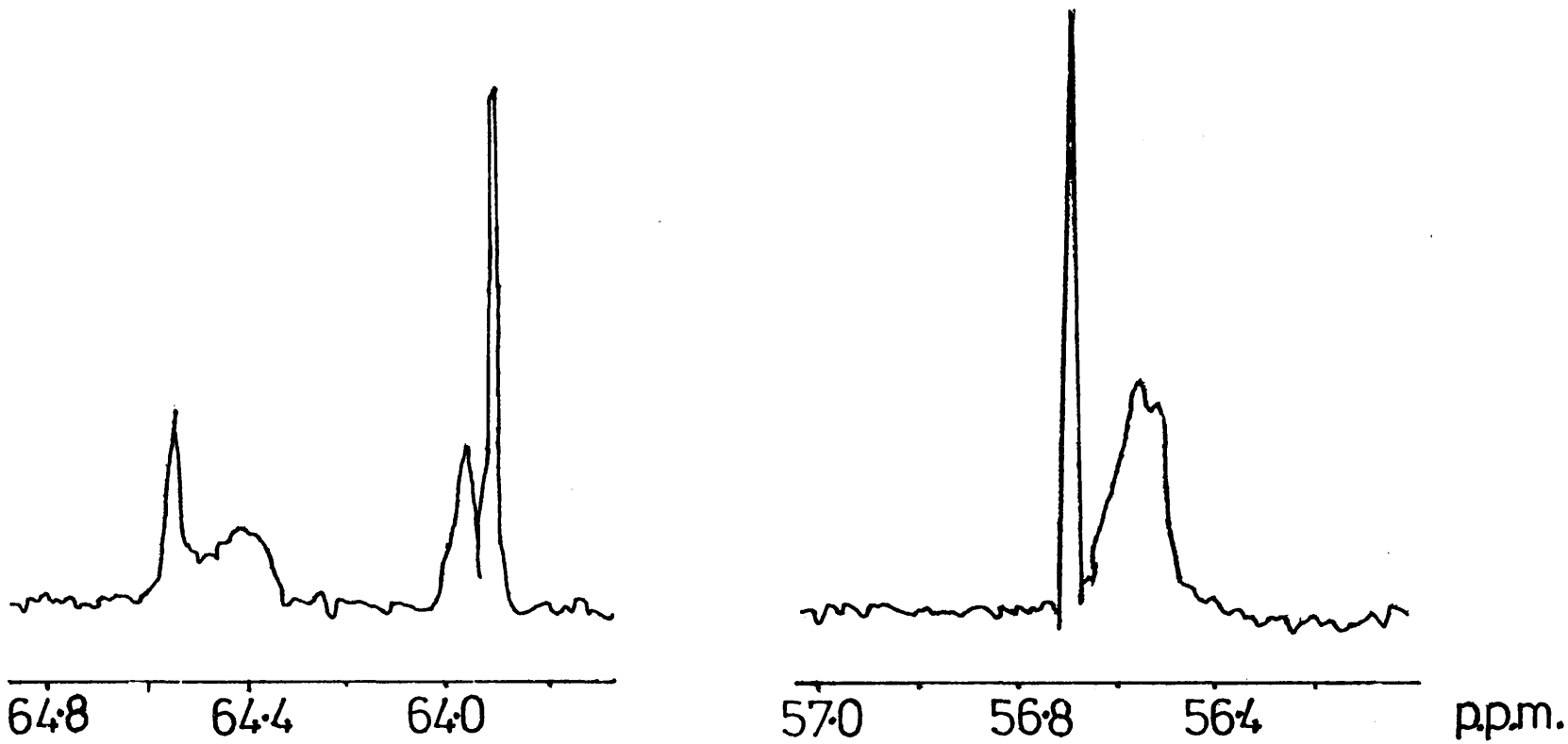
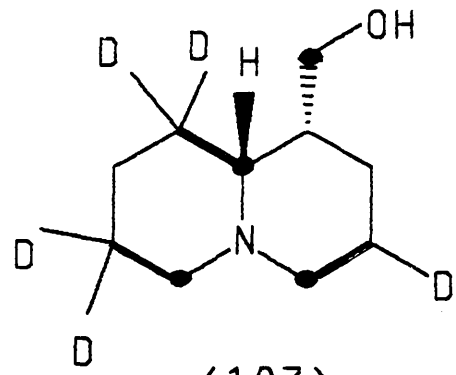


Figure 8. Expansion of ^{13}C n.m.r. spectrum of lupinine.



being two deuterium atoms attached to C-10, with ^{13}C-^{13}C coupling occurring between C-10 and C-6, through nitrogen. Unfortunately in the spectrum of free lupinine, the signals for C-4 and C-6 are at the same shift value. The peaks observed here therefore are the combination of the signals from these two carbon atoms. There appears to be two deuterium atoms associated with the normal carbon signal. Whether both the carbon atoms are attached to one deuterium is uncertain. By reference to both spectra, that of the hydrochloride salt and that of the free base, it is reasonable to propose the labelling pattern shown below in (107).

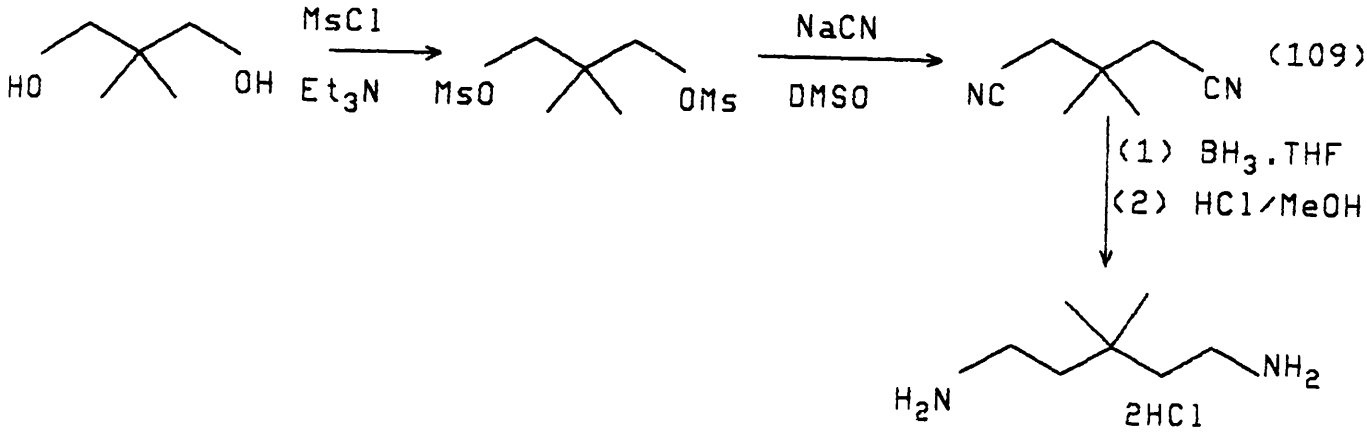


(107)

3.6 Biosynthesis of Analogues of Quinolizidine Alkaloids

Many research workers have found it desirable to study the biosynthesis of a natural product using a precursor analogue, as described in Chapter 2. It was decided to prepare two analogues of cadaverine and feed them to plants producing quinolizidine alkaloids in order to study the fate of unnatural precursors.

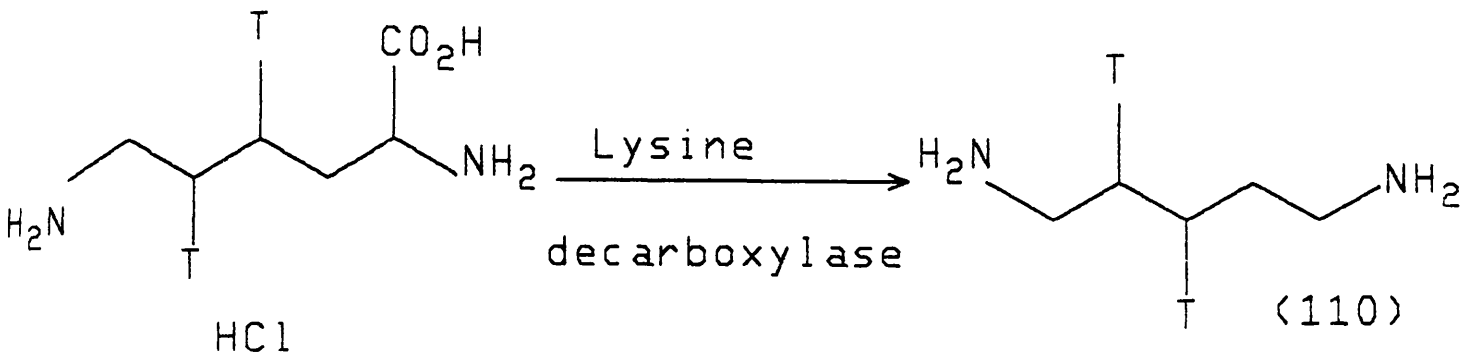
3,3-Dimethylcadaverine dihydrochloride (108) was synthesised by the route shown in Scheme 39.



Scheme 39

Mesylation of 2,2-dimethylpropane-1,3-diol in 92% yield, followed by displacement of the mesyl groups by sodium cyanide afforded the dinitrile (109) in 43% yield. The displacement step was troublesome; the S_N2 reaction was slow due to steric hindrance. A diborane reduction produced the diamine, and acidification gave 3,3-dimethylcadaverine dihydrochloride (108) in 64% yield. ^{14}C-Labelled 3,3-dimethylcadaverine dihydrochloride was prepared in analogous fashion using sodium ^{14}C-cyanide in the displacement reaction.

Samples of ^{14}C-labelled 3,3-dimethylcadaverine were fed with ^{3}H-labelled cadaverine dihydrochloride in order to compare the incorporation level of the unnatural precursor with that of the natural one. ^{3}H-Labelled cadaverine (110) was prepared by decarboxylation of [4,5-^{3}H]lysine monohydrochloride by lysine decarboxylase enzyme in water, at 37°C (Scheme 40).



Scheme 40

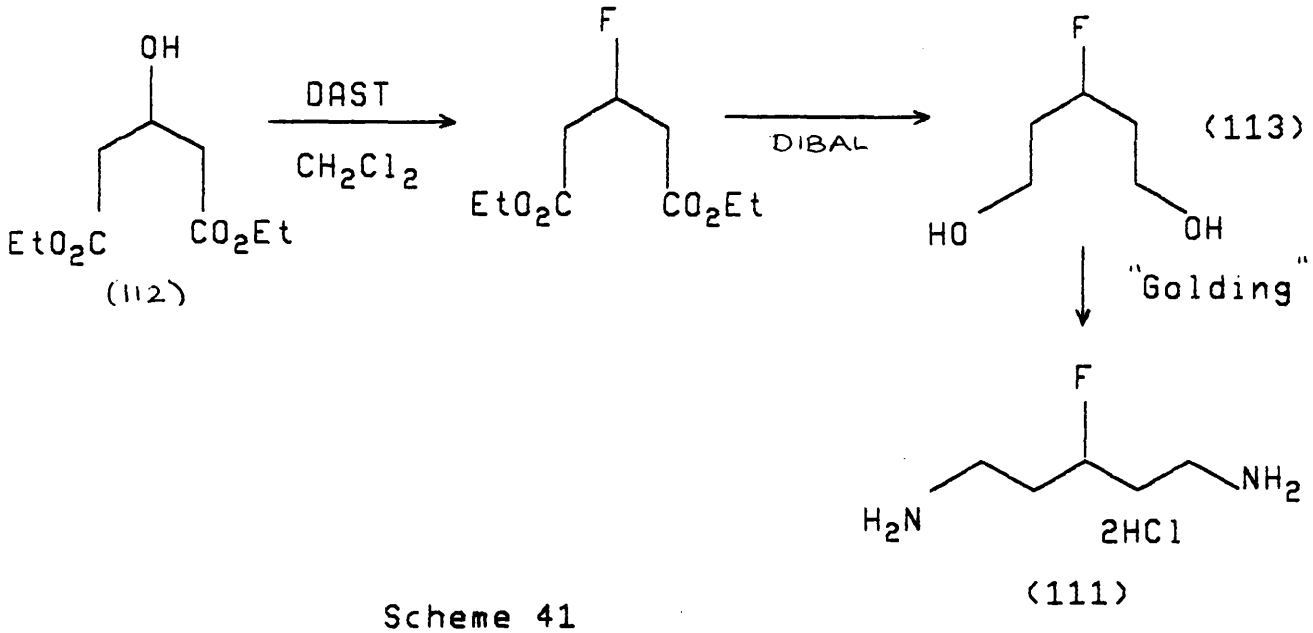
The ^{14}C- and ^{3}H-labelled cadaverines were fed to five species - Baptisia australis, Sophora microphylla, Anagyris foetida, Lupinus luteus and Lupinus polyphyllus. Two plants from each species were used in each experiment. The wick method of feeding was employed for S. microphylla, A. foetida and B. australis while L. luteus and L. polyphyllus were fed by the xylem pricking method. The precursors were fed over 2 days and harvested a week later. The alkaloidal mixture was extracted in the usual way, and incorporation levels determined by scintillation counting. The incorporation levels attained are detailed in Table 6. The incorporation levels show that the unnatural precursor was incorporated 50-100% as well as the cadaverine. The highest incorporations were achieved with L. luteus, A. foetida and S. microphylla plants. It was, therefore, decided to bulk-feed 3,3-dimethylcadaverine to L. luteus and S. microphylla plants, extract the resulting alkaloid mixture and hopefully isolate new analogues of quinolizidine alkaloids.

Table 6

Plant	^{3}H Total Incorporation (Cadaverine) %	^{14}C Total Incorporation (3,3-Dimethyl-cadaverine) %
L. luteus	7.36	3.38
L. polyphyllus	2.09	2.03
A. foetida	8.17	4.10
S. microphylla	14.01	7.55
B. australis	1.22	1.31

A measured amount of 3,3-dimethylcadaverine dihydrochloride (109) was mixed with a known quantity of radioactive 3,3-dimethyl-cadaverine. The mixture was fed to eight S. microphylla plants and to 16 L. luteus plants. The plants were fed over a period of 10 days then left for a further 14 days before harvesting. The plants were then macerated, and the alkaloids extracted using the normal procedure. Total incorporation levels of 1.8% for S. microphylla and of 1.8% for L. luteus were observed. Analysis of the alkaloidal material by t.l.c. revealed that both samples were complex mixtures. Radioscans of the two mixtures were recorded and revealed that radio-activity was spread over the plates, with little increased concentration observed on portions corresponding to alkaloids. Separation of the complex mixtures of alkaloids would be extremely difficult, so investigations into the manufacture of quinolizidine alkaloids using this precursor were not continued.

A second cadaverine analogue was prepared for studying quinolizidine alkaloid biosynthesis - 3-fluorocadaverine dihydrochloride (111). This compound was chosen because the presence of F in metabolites should be easy to observe by ^{19}F n.m.r. spectroscopy. Scheme 41 shows the chosen route to the compound.



Scheme 41

Diethyl 3-hydroxyglutar ate (112) was fluorinated using diethylaminosulphur trifluoride (DAST). The resulting fluorodiester was reduced with DIBAL to 3-fluoropentane-1,5-diol (113). The "Golding" reaction was then performed on the fluoro-diol - the substrate was dissolved in THF and hydrazoic acid, di-isopropylazodicarboxylate and an excess of triphenylphosphine was added. After the reaction mixture was heated for 4h, dilute acid was added to produce hydrolysis and work-up yielded 3-fluorocadaverine dihydrochloride (111). The precursor was analysed by ^{19}F n.m.r. spectroscopy, and a peak at -184.5 p.p.m. was noted.

3-Fluorocadaverine dihydrochloride (48 mg) was fed alongside ^{3}H-labelled cadaverine dihydrochloride to eight L. luteus plants. The cadaverine was administered to the plants over 6 days, at which point the plants looked sick - stems were drooping and some of the leaves had turned brown. The feeding was therefore stopped. The plants were harvested seven days after termination of feeding. The alkaloidal mixture was isolated in the usual manner, and a total incorporation of 5.94% was noted. T.l.c. analysis of the extract showed two large spots at Rf 0.10 and 0.28 corresponding to sparteine and lupinine, plus two small spots at Rf 0.48 and 0.55, possibly corresponding to analogues of the alkaloids. No peaks were observed in the fluorine n.m.r. spectrum of the extract, however. This suggests that although the cadaverine was being metabolised by the plant, the 3-fluorocadaverine was not converted into quinolizidine alkaloids. The unnatural precursor may not have been metabolised at all by the plant, or it may have blocked the biosynthetic pathway at a certain stage in the route - this remains unclear. Again, no definite conclusions can be drawn from the results of the feeding experiment.

Future experiments using cadaverine analogues should incorporate the following features:-

(i) A label on the cadaverine which is easy to identify, such as fluorine. The methyl group of 3,3-dimethylcadaverine could not be noticed in the spectra of the alkaloid extracts.

(ii) An easy route to the corresponding radiolabelled precursor is desirable, so that a valid incorporation level can be recorded.

(iii) Plants with simple alkaloidal mixtures should be employed to reduce the number of possible alkaloid analogues.

(iv) Symmetrical precursors should be used at first; again to lessen the number of possible products.

If all of the above suggestions were utilised, there would be good prospects of producing quinolizidine alkaloid analogues.

3.7 Physiological Experiments

It was felt necessary to carry out some background experiments on the plants used to produce the quinolizidine alkaloids matrine (11), anagyrine (8), N-methylcytisine (50), cytisine (7), lupinine (4) and sparteine (5). These will be described in turn:-

3.7.1 Incorporation of ^{3}H-Labelled Cadaverine in Comparison with ^{14}C-Labelled Cadaverine

It is possible to "spike" an analogue feed with either ^{14}C- or ^{3}H-labelled cadaverines, and both methods are used commonly. It was decided to compare the incorporation of the two radioactive precursors so that a comparison could be made at any time. [2,3-^{3}H]-Cadaverine dihydrochloride (110) was prepared by decarboxylation of [4,5-^{3}H]lysine monohydrochloride with lysine decarboxylase (Scheme 40). Similarly, ^{14}C-labelled cadaverine was synthesised by decarboxylation of ^{14}C-labelled lysine with lysine decarboxylase.

The cadaverines prepared were fed in a ^{3}H:^{14}C ratio of 15:1 to three plant species - L. luteus, S. microphylla and A. foetida,

one plant of each species being used each time. The compound was fed at one administration, and after a further 13 days the plants were macerated and the alkaloids were extracted. The crude alkaloidal mixtures were submitted for scintillation counting and the results are shown in Table 7.

Table 7

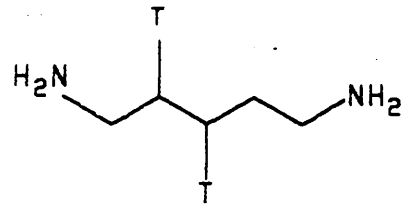
Plant	Total ^{3}C Incorporation	Total ^{14}C Incorporation	New $^{3}H:^{14}C$ Ratio
S. microphylla	2.69	4.19	9.6:1
L. luteus	10.00	12.35	12.3:1
A. foetida	20.68	33.22	9.4:1

The crude mixtures were separated by preparative t.l.c. on silica plates. Samples of the individual alkaloids were then analysed, and the new ratio calculated for each compound. The results are shown in Table 8.

Table 8

Plant	Alkaloid	New Ratio of $^{3}H:^{14}C$
S. microphylla	Matrine	11.0:1
	Anagyrine	10.2:1
	N-methylcytisine	7.7:1
	Cytisine	5.7:1
A. foetida	Anagyrine	9.3:1
	N-methylcytisine	8.0:1
L. luteus	Lupinine	9.4:1
	Sparteine	5:1

The results indicate that the ^{3}H-labelled cadaverine is less well incorporated than the ^{14}C-precursor - that is, some tritium is lost in the biosynthetic pathway to the alkaloids. On inspection of the ^{3}H-labelled precursor (110), it was seen that the tritium label could easily



(110)

2HCl

be lost if imine-enamine tautomerisms operate on the route to the alkaloids. The amount of tritium lost will depend on the number of times this equilibrium operates in the pathway, and will also be dependent upon the stereochemistry of the tritium atom when the tautomerisation occurs. Isotope effects may also be important.

This experiment, therefore, enables both types of radiolabel to be used with easy comparison of incorporation.

3.7.2 Incorporation and Time Study with S. microphylla

In most biosynthetic experiments, the plants are fed over a period of 10-14 days and harvested after a further 10-14 days. Since little work had been carried out on S. microphylla plants, it was considered useful to study the incorporations of the precursor after various time intervals.

Six plants were wick fed with [2,3-^{3}H]cadaverine dihydrochloride (110) and left for 1, 2, 4, 8, 16 and 32 days before harvesting and extracting the alkaloids in the usual fashion. The incorporations measured are shown in Table 9.

Table 9

Number of Days Before Harvesting	Total Incorporation (%)
1	2.81
2	2.48
4	1.98
8	1.78
16	4.67
32	1.55

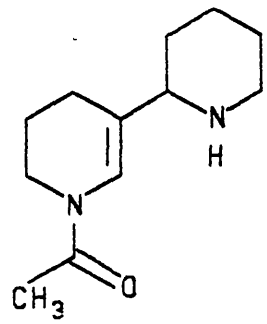
The results reflect the alkaloid turnover taking place in the plants. As cadaverine is metabolised to alkaloids, the incorporation increases, reaching a maximum after about two weeks. The incorporation falls again as the radioactive alkaloids are broken down and the radioactivity is dissipated. A similar pattern was observed by Wink. It is clear from the results that the optimum incorporation occurs after around two weeks: this corresponds to the best time to harvest the plants after feeding.

CHAPTER FOUR

BIOSYNTHESIS OF AMMODENDRINE

4.1 Introduction

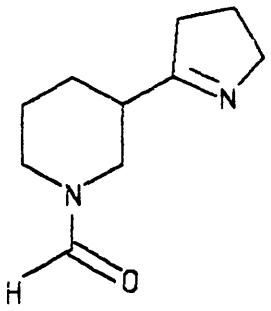
Ammodendrine (16) has been found as a minor alkaloid alongside quinolizidine alkaloids in 28 plant species.[99]



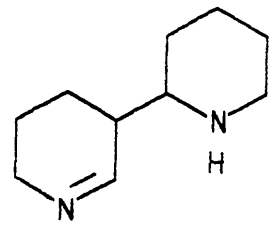
(16)

Cadaverine is a precursor for ammodendrine and investigations into its metabolism showed that ^{14}C- and ^{2}H-labelled cadaverines were incorporated into both rings of the compound.

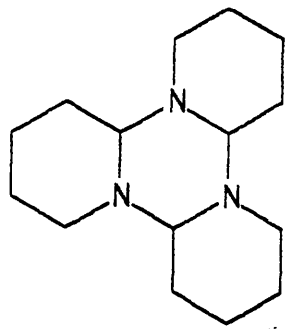
Studies of the biosynthesis of ammodendrine have been made in cell-free extracts by Wink and Witte. They found that when cadaverine was incubated with cell-free extracts of Lupinus arboreus and Pisum sativum and pyruvate, ammodendrine was formed. In a similar experiment employing (R)-[1-^{2}H]cadaverine, ammodendrine was again formed, but its molecular ion was two units higher. In these experiments, other products, namely smipine (114), tetrahydroanabasine (23) and tripiperideine (115) were also formed.



(114)



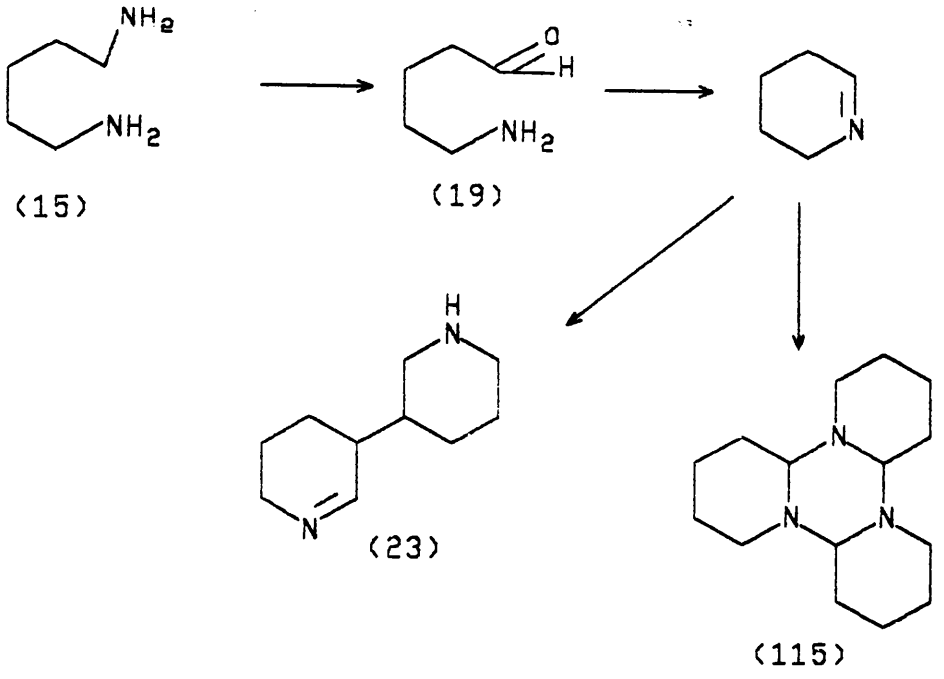
(23)



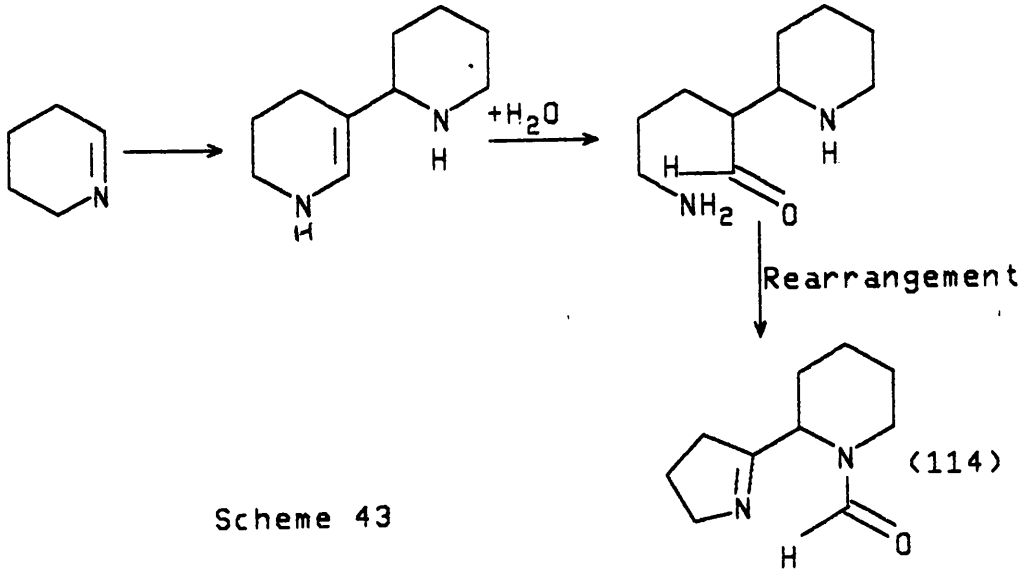
(115)

The biosynthesis of the alkaloids is thought to occur via the oxidation of cadaverine to 5-aminopentanal (19), which is in equilibrium with Δ^1-piperideine. This step is probably catalysed by diamine oxidase. Under physiological conditions, spontaneous dimerization to tetrahydroanabasine (23) or trimerization to α-tripiperideine (115) can occur, thus explaining the presence of these two compounds in the cell-free extracts (Scheme 42).

Acetylation of N-1 to form ammodendrine may be catalysed by pyruvate dehydrogenase[99], since acetyl-CoA is not present in the reaction mixtures. Smipine may also be formed from tetrahydroanabasine by hydrolysis of the imine, followed by an unusual rearrangement Scheme 43).



Scheme 42



Scheme 43

Smipine was found to be optically inactive, probably as a result of enamine-imine tautomerism.

It was decided to extend these studies by feeding enantiomerically labelled cadaverines and analysing the products to find out if the processes involved in the biosynthesis of ammodendrine are stereospecific. The cadaverines used were (R)- and (S)-[1-^{2}H]cadaverine, prepared as previously described. The deuterium content was estimated to be ca. 90% for the (R)- and ca. 85% for the (S)-[1-^{2}H]cadaverine. This work was performed under the supervision of Professor M. Wink at Munich University in June 1988, and the GC-MS data were collected at Braunsweig University by Dr. L. Witte.

4.2 Investigations into the Biosynthesis of Ammodendrine

Ammodendrine was biosynthesised from (R)- and (S)-[1-^{2}H]-cadaverines using four different systems:-

(i) Complete leaf and petiole of L. polyphyllus

(ii) Leaf discs of 1 mm width from L. polyphyllus plants

(iii) Two different DAO enzymes - from L. arboreus and P. sativum

(iv) Enzyme extracts from L. polyphyllus plants.

After incubation with the cadaverines for the specified time, the reaction mixtures were worked up and the alkaloids were extracted. Capillary GLC was carried out on the alkaloidal mixtures at Munich and GC-MS analysis was performed at Braunsweig.

Figure 9. GLC trace from complete leaf and petiole.

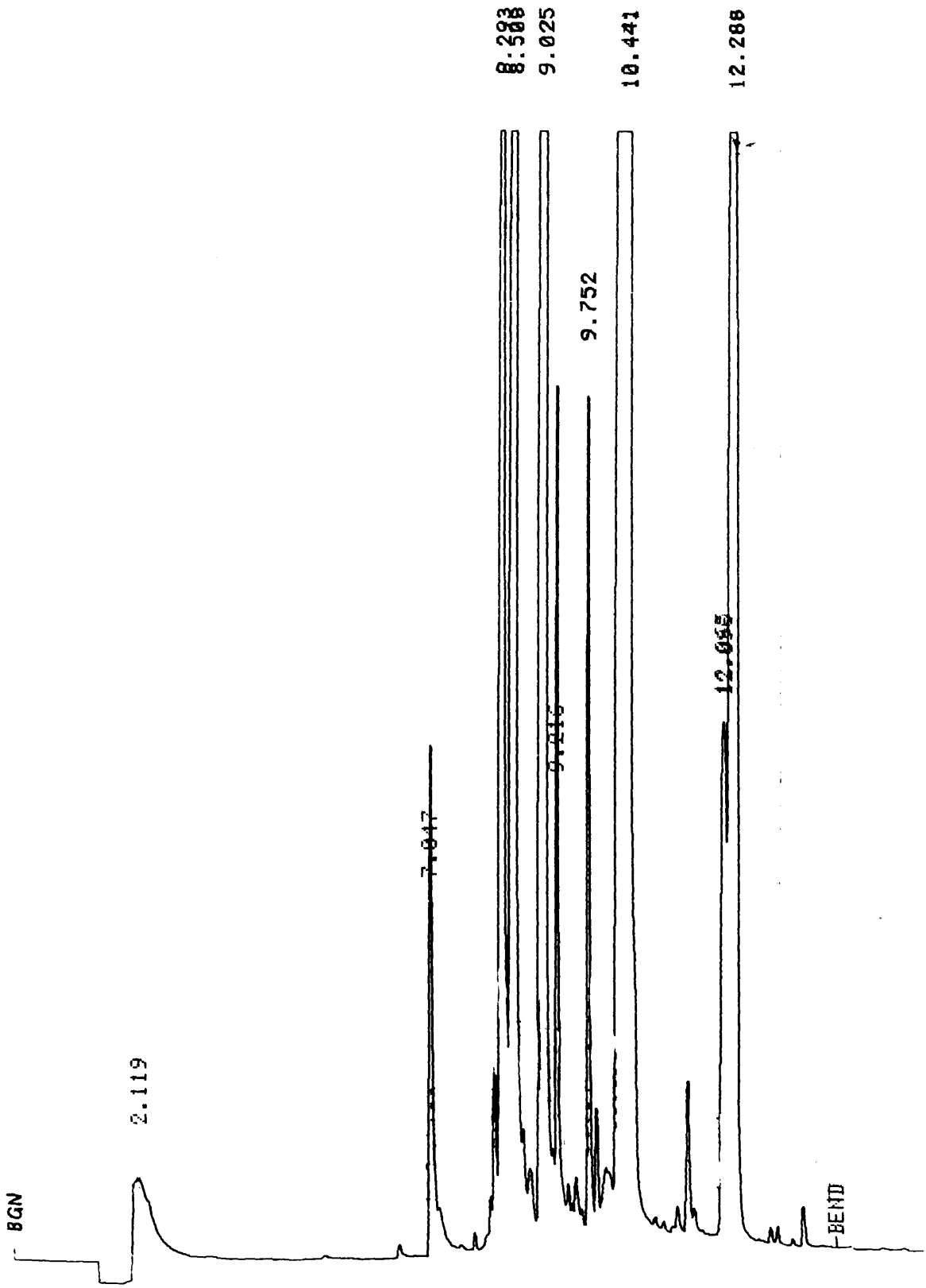


Figure 10. GLC trace from L.polyphyllus enzyme extract.

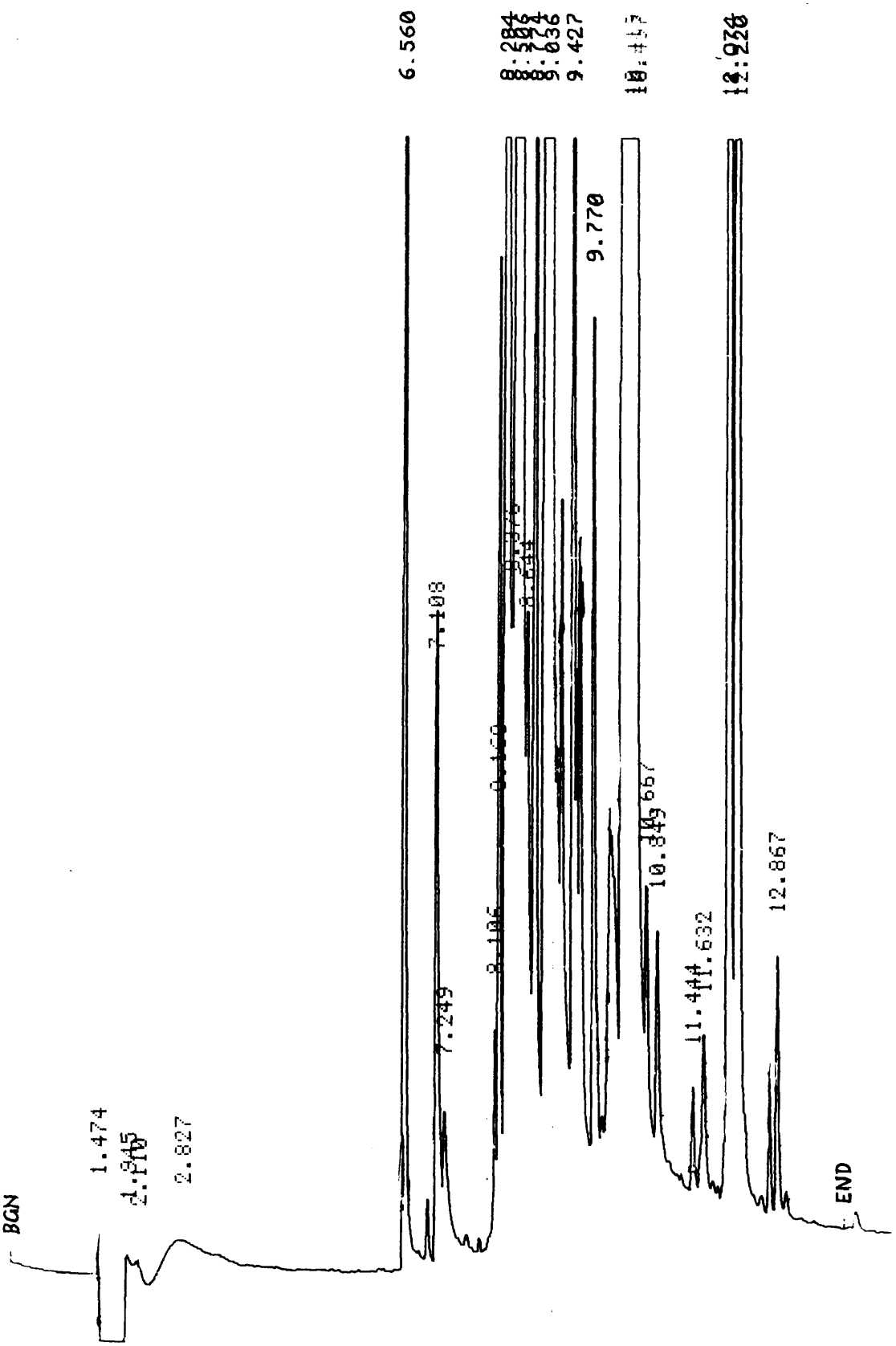


Figure 11. GLC trace from DAO enzyme.

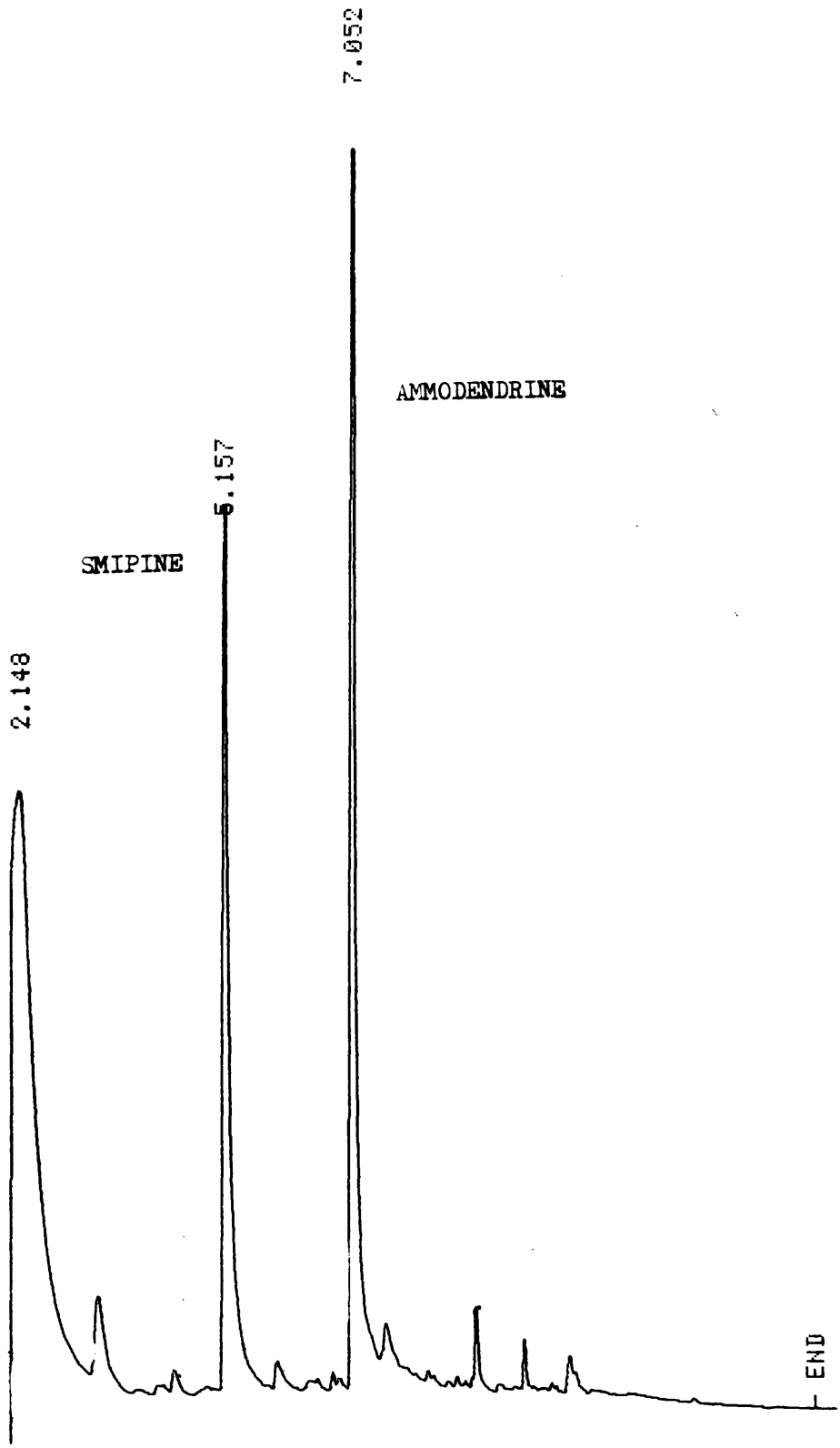
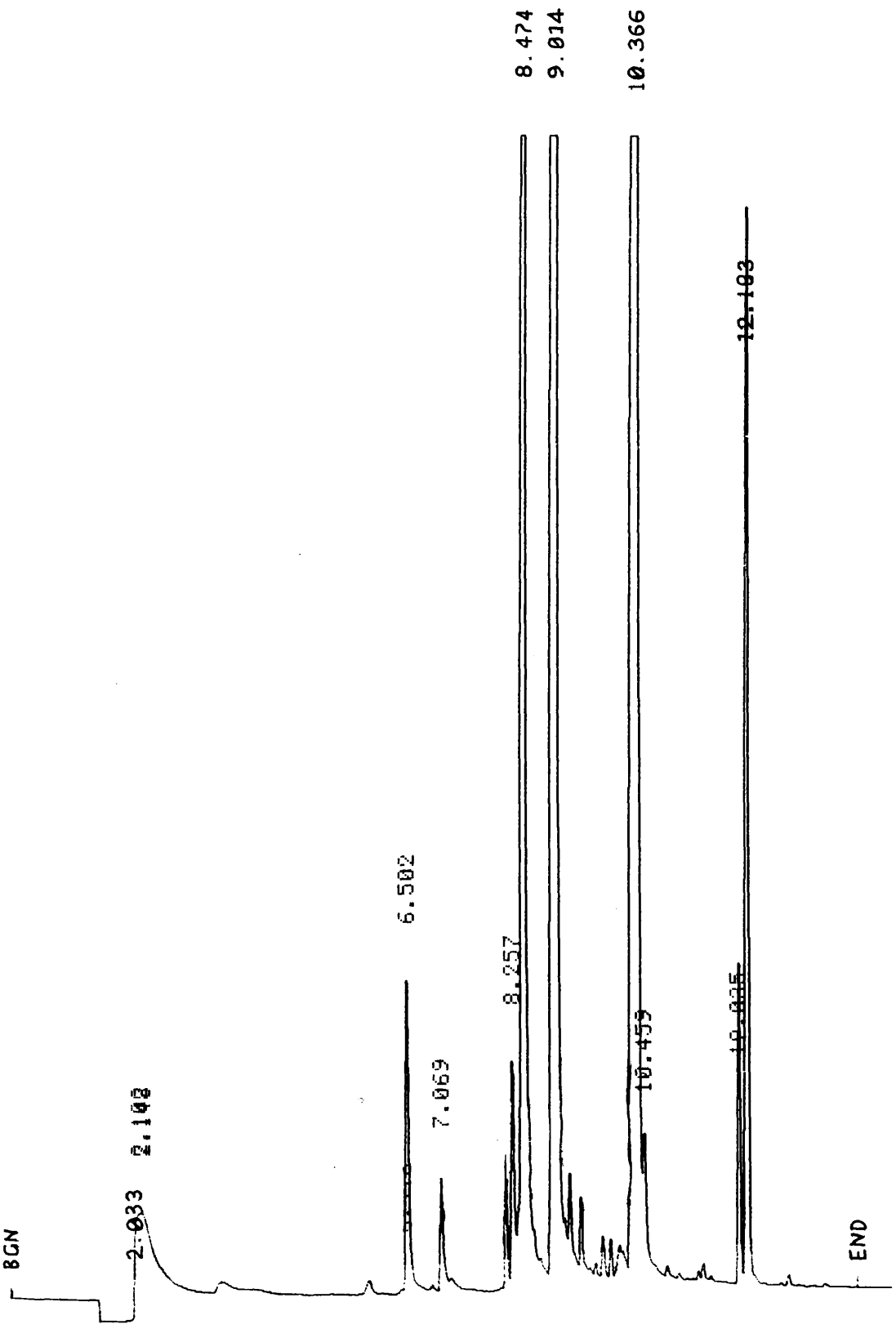


Figure 12. GLC trace from leaf discs.



The GLC traces from the complete leaf and petiole and the L. polyphyllus enzyme experiments show that relatively little ammodendrine and smipine are formed (Figs. 9 and 10). The DAO enzymes and the leaf discs, however, produced ammodendrine and smipine as the major products (Figs. 11 and 12). It is the results of the GC-MS of the alkaloids formed using DAO enzymes that are used in the calculations since they are least affected by dilution with endogenous unlabelled material.

GC-MS data were collected under standard conditions.[100] In addition, MS scans taken from various parts of each GLC peak were checked for homogeneity to ensure that there was no fractionation of isotopically labelled species in the GLC. Changes in the relative peak intensities were shown to be $<$ 10% by repeated MS scans. The intensity values for M-2, M-1, M, M+1, M+2 and up to M+3 (where M = MW of unlabelled metabolite) were recorded for each metabolite. These figures were subsequently corrected for contributions to the peaks by A+1 and A+2 ions (where A = mass of ion under study) by the following formula:-[101]

$$\frac{(A+1)^2}{A^+} = 1.1\% \times \text{number of C atoms} + 0.36\% \times \text{number of N atoms}$$

$$\frac{(A+2)^2}{A^+} = \frac{(1.1 \times \text{number of C atoms})^2}{200}\% + 0.20\% \times \text{number of O atoms}$$

These corrections were applied in turn to each peak M, M+1, M+2, M+3 to arrive at values for the peak intensities for each metabolite. Analysis of the MS of unlabelled metabolites by this method showed that there

were no interfering ions present around the mass peak area.

Richards and Spenser have shown that oxidation of cadaverine by pea seedling DAO takes place without detectable intramolecular primary isotope effect.[102]

The GC-MS data for each metabolite obtained from the DAO experiments will be discussed in turn. Exactly analogous results were obtained from the leaf and petiole experiments.

4.2.1 Tetrahydroanabasine

The traces from the GC-MS from the (R)- and (S)-[1-^{2}H]-cadaverine feeds are shown in Figs. 13 and 14, while the peak intensities in their uncorrected and corrected forms are shown in Table 10 for the (R)-[1-^{2}H]cadaverine feed.

Table 10

Mass Number	Peak Intensity	Corrected Peak Intensity	Percentage	Estimate for 90% Deuterium Content
164(M-2)	58	58	0.5	0
165(M-1)	189	182	1.5	0
166(M)	1152	1131	9.5	1
167(M+1)	2892	2760	23.2	18
168(M+2)	7440	7110	59.8	81
169(M+3)	1506	658	5.5	0

The major peak at M+2 suggests that both oxidations of cadaverine involve retention of the pro-R hydrogens. Since the deuterium content

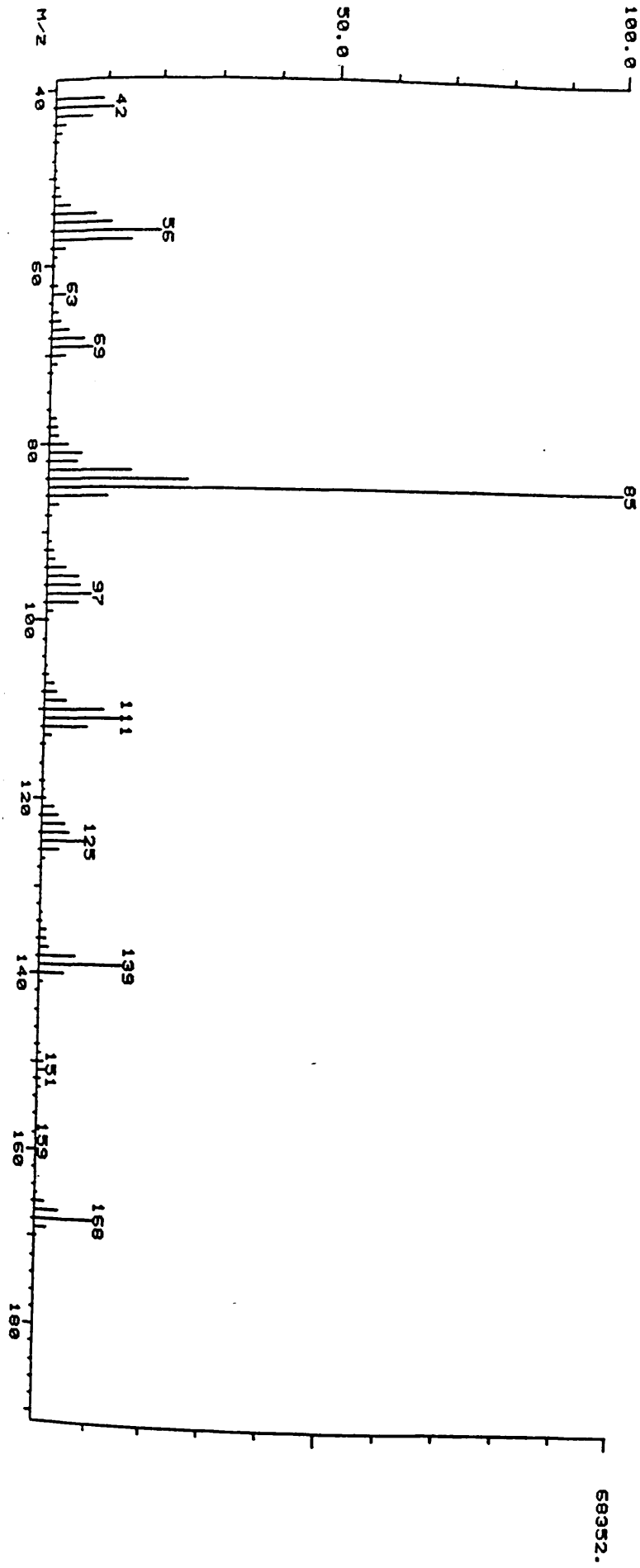
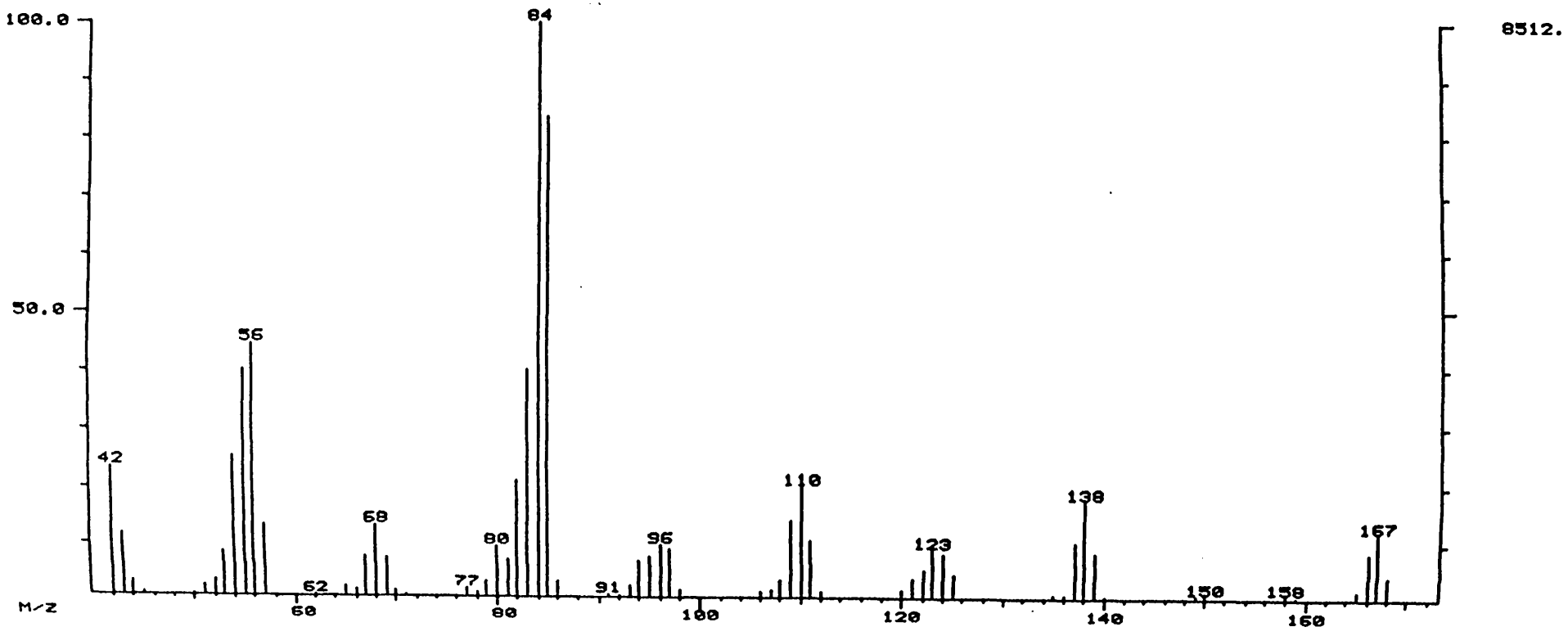


Figure 13. GC-MS spectrum of tetrahydroanabasine derived from (R)-[1-^{2}H]-cadaverine.

Figure 14. GC-MS spectrum of tetrahydroanabasine derived from (S)-[1-^{2}H]- cadaverine.



of the cadaverine is estimated to be 90% then the calculated distribution of the M, M+1 and M+2 peaks would be 1%, 18% and 81%. The experimental values are lower, indicating that some deuterium has been lost. Two piperideine units couple to form tetrahydroanabasine and racemisation possibly occurs after its production. These processes may remove a pro-R hydrogen present after the initial oxidations of cadaverine have taken place.

The GC-MS data from the (S)-[1-^{2}H]cadaverine feed is shown in Table 11.

Table 11

Mass Number	Uncorrected Peak Intensity	Corrected Peak Intensity	Percentage	Calculated For 85% x 50%
166(M)	179	179	40.6	33.1
167(M+1)	231	210	47.6	48.9
168(M+2)	68	52	11.8	18.1

If the pro-S hydrogen is lost from cadaverine on each oxidation and the kinetic isotope effect is negligible, then 50% of the deuterium in each cadaverine will be lost. Taking into account the 85% deuterium content of the cadaverine fed, then the calculated values of M = 33.1, M+1 = 48.9 and M+2 = 18.1 are in reasonable agreement with the experimental values.

Figure 15. GC-MS spectrum of ammodendrine derived from (R)-[1-^{2}H]- cadaverine.

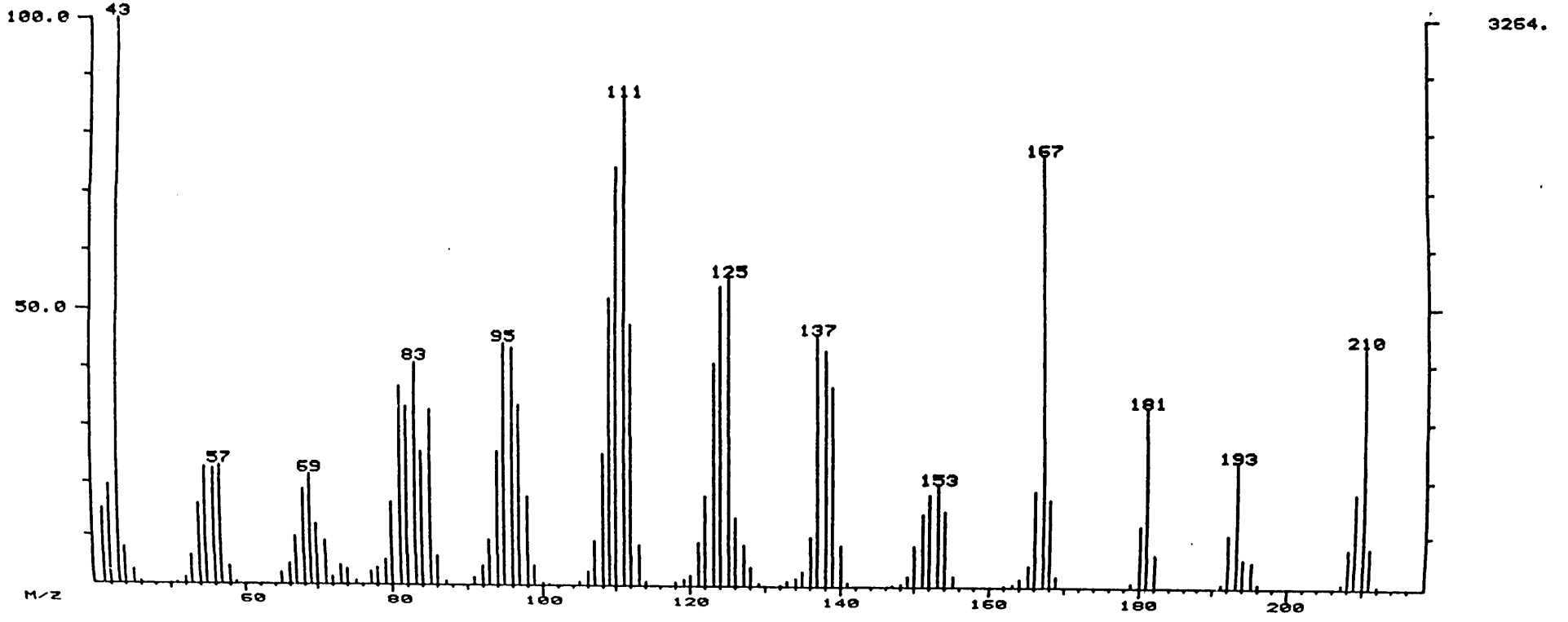
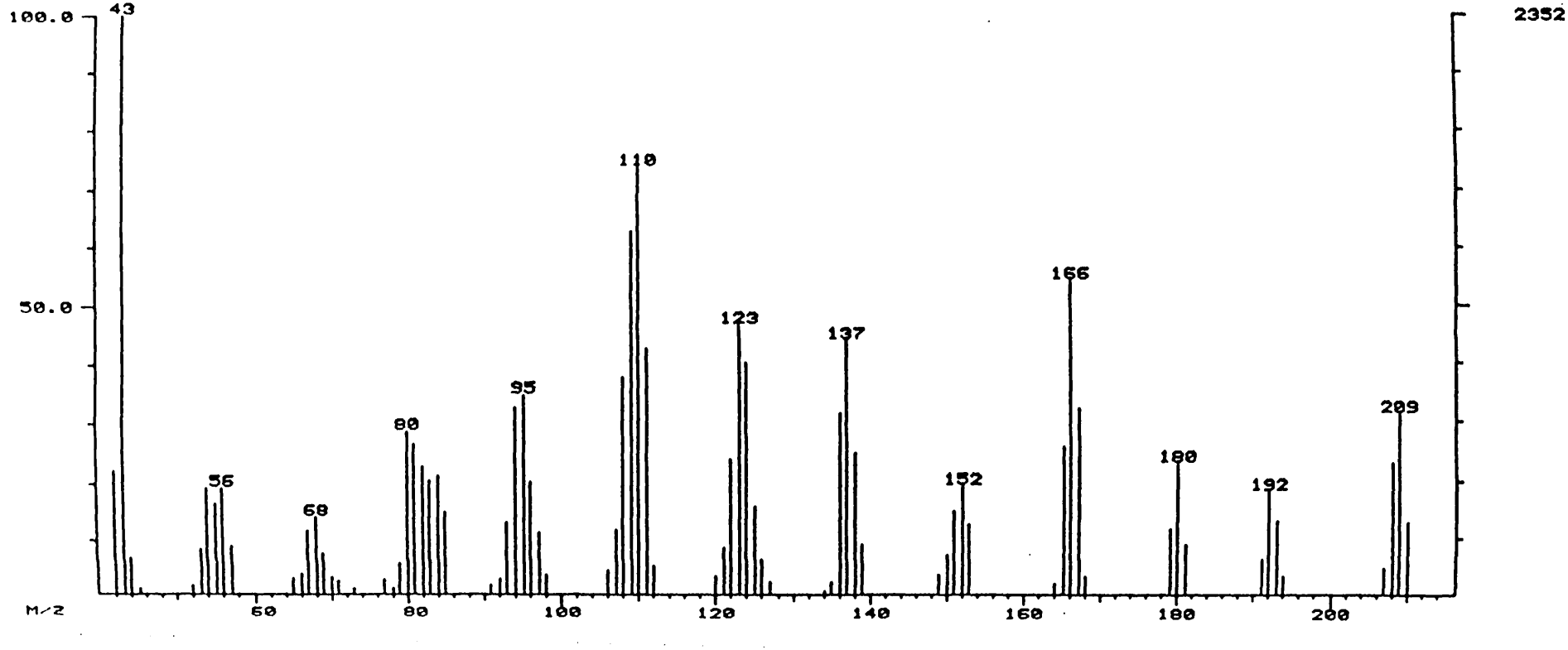


Figure 16. GC-MS spectrum of ammodendrine derived from (S)-[1-^{2}H]- cadaverine.



4.2.2 Ammodendrine

The results from the feeding experiments with ammodendrine are similar to those obtained from tetrahydroanabasine. Figs. 15 and 16 show the GC-MS traces obtained after feeding (R)- and (S)-[1-^{2}H]-cadaverine respectively. Table 12 shows the results of the GC-MS analysis from feeding the (R)-precursor.

Table 12

Mass Number	Uncorrected Peak Intensity	Corrected Peak Intensity	Percentage	Calculated for 90%
207(M-1)	27	27	1.2	0
208(M)	243	239	10.9	1
209(M+1)	578	545	24.8	18
210(M+2)	1408	1330	60.4	81
211(M+3)	252	61	2.8	0

The values obtained are similar to those obtained earlier by Wink and Witte. Loss of deuterium may again occur during coupling or racemisation during the formation of ammodendrine.

The figures obtained after feeding the (S)-precursor, shown in Table 13, are also similar to those of tetrahydroanabasine and agree well with the calculated values. This supports the theory that the pro-S hydrogen is lost on oxidation of cadaverine.

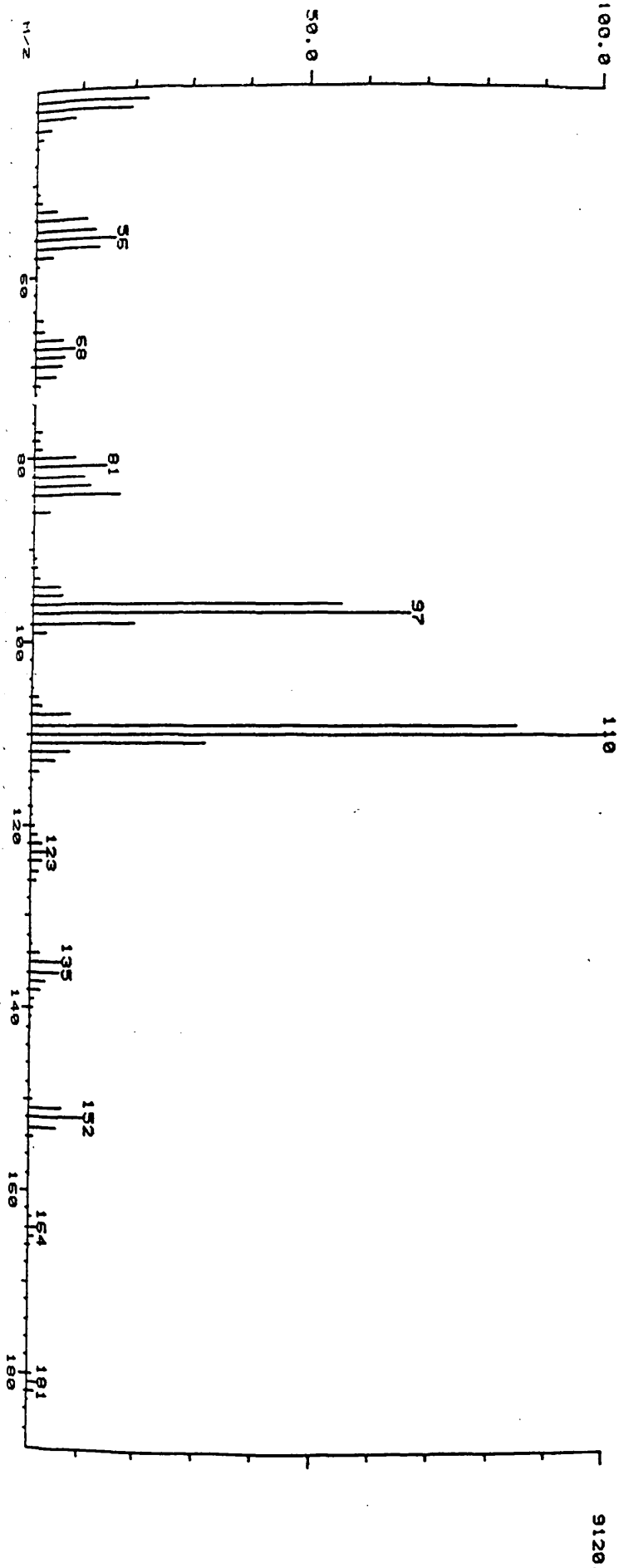


Figure 17. GC-MS spectrum of smipine derived from (R)-[1-^{2}H]-cadaverine.

Table 13

Mass Number	Uncorrected Peak Intensity	Corrected Peak Intensity	Percentage	Calculated For 85% x 50%
207(M-1)	28	28	6.0	0
208(M)	158	154	32.8	33.1
209(M+1)	251	229	48.9	48.8
210(M+2)	92	58	12.4	18.1

4.2.3 Smipine

The data from the GC-MS of smipine are shown in Figs. 17 and 18 for (R)- and (S)-[1-^{2}H]cadaverine feeds, respectively. The mass distributions after feeding the (R)-precursor, shown in Table 14 indicate that around one half of the deuterium is lost on route to smipine.

Table 14

Mass Number	Uncorrected Peak Intensity	Corrected Peak Intensity	Percentage	Calculated For 90% x 50%
180(M)	63	63	19.4	20.2
181(M+1)	173	165	50.9	49.5
182(M+2)	114	93	28.7	30.2
183(M+3)	16	3	0.9	0

Since the pro-R hydrogens are not lost in the cadaverine oxidation, the loss must occur later in the pathway. As tetrahydroanabasine is on the smipine pathway, some deuterium will be lost in the formation and possible racemisation of tetrahydroanabasine (as indicated by previous results). Further loss of deuterium, however, must occur in the rearrangement processes to form smipine.

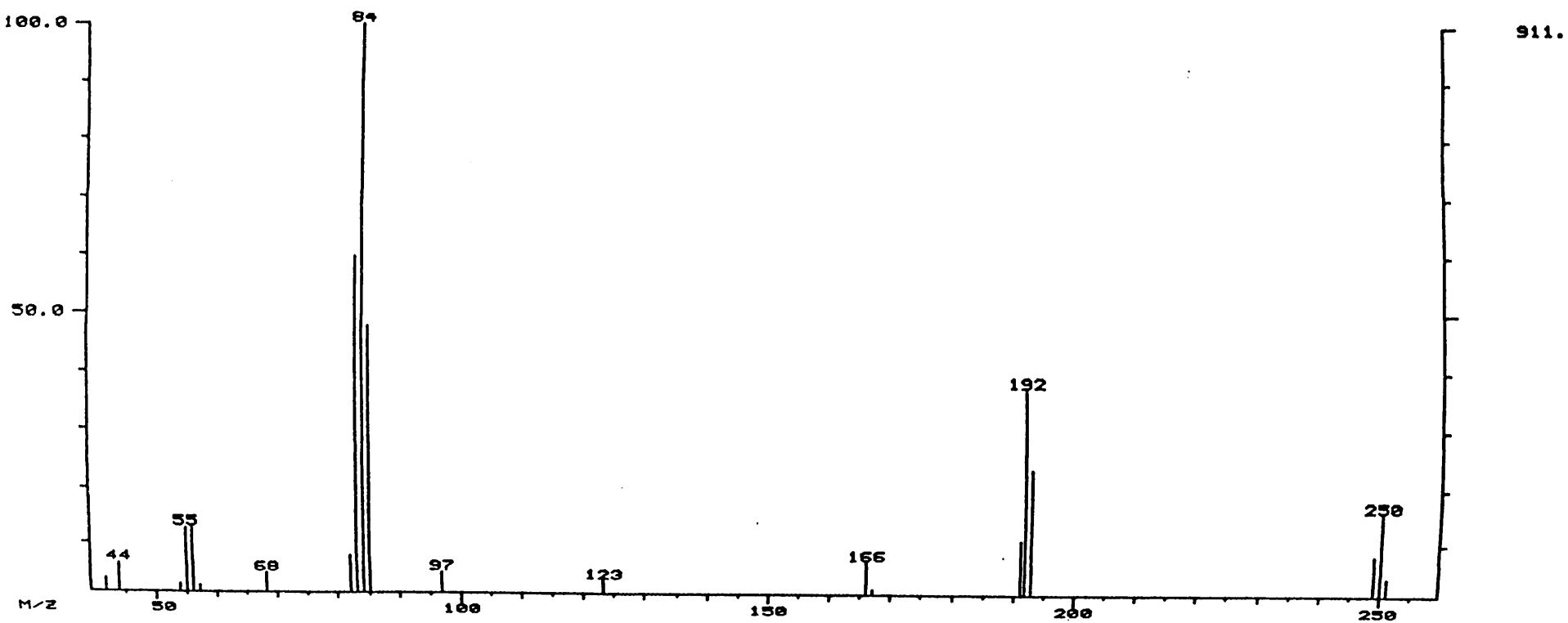
The results from the (S)-[1-^{2}H]cadaverine fed to smipine are shown in Table 15. The calculated values assuming loss of 50% of the deuterium during the initial oxidations are in excellent agreement, supporting the assertion that the pro-S hydrogen is lost on oxidation of cadaverine. The results are also consistent with there being little intramolecular primary kinetic isotope effect operating in the oxidations. No further loss of deuterium occurs in the formation of smipine (cf. the (R)-[1-^{2}H]cadaverine case) since the positions involved would have already lost their deuterium atoms in the oxidations.

Table 15

Mass Number	Uncorrected Peak Intensity	Corrected Peak Intensity	Percentage	Calculated For 85% x 50%
180(M)	71	71	30.9	33.1
181(M+1)	124	115	50.0	48.9
182(M+2)	59	44	19.1	18.1

Figure 19. GC-MS spectrum of tripiperideine derived from (R)-[1-^{2}H]-cadaverine.

Figure 20. GC-MS spectrum of tripiperideine derived from (S)-[1-^{2}H]-cadaverine.



4.2.4 Tripiperideine

The GC-MS traces obtained after feeding the cadaverine precursors (35) and (36) are shown in Figs. 19 and 20. Table 16 presents the data from the (R)-[1-^{2}H]cadaverine feed.

Table 16

Mass Number	Uncorrected Peak Intensity	Corrected Peak Intensity	Percentage	Calculated For 90% Deuterium Content
249(M)	0	0	0	0.1
250(M+1)	122	122	3.7	2.7
251(M+2)	736	714	21.9	24.3
252(M+3)	2524	2394	73.3	72.9
253(M+4)	475	37	1.1	0

There is again excellent correlation between the experimental values and those calculated with a strong M+3 peak, confirming that it is the pro-R hydrogens that are retained in the oxidation of three cadaverine units. It also indicates that there has been little dilution with endogenous unlabelled cadaverine or tripiperideine. The results obtained after feeding (S)-[1-^{2}H]cadaverine are detailed in Table 17. The values indicate there is a much greater deuterium loss than is expected from loss due to the oxidation. The reasons for this high loss of deuterium are, at present, unclear.

Table 17

Mass Number	Uncorrected Peak Intensity	Corrected Peak Intensity	Percentage	Calculated For 85% x 50%
249(M)	70	70	34.0	19.0
250(M+1)	142	129	62.6	42.2
251(M+2)	31	7	3.4	31.2
252(M+3)	0	0	0	7.7

4.2.5 Conclusions

Several conclusions can be drawn from the results of the feeding experiments with (R)- and (S)-[1-^{2}H]cadaverines:

(i) It is the pro-S hydrogen that is lost, and the pro-R hydrogen that is retained in all processes involving the conversion of $-CH_2NH_2$ to -CHO.

(ii) The resemblance of the results obtained with DAO enzyme to those from lupin petioles indicates that similar enzymatic processes are operating in both systems.

(iii) The oxidation of cadaverine in the systems takes place without detectable intramolecular primary kinetic isotope effect, supporting the findings of Richards and Spenser.

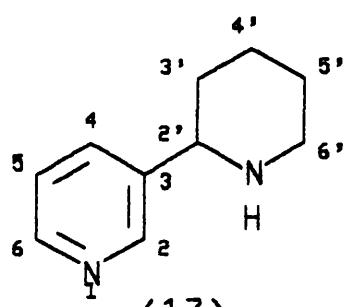
CHAPTER FIVE

BIOSYNTHESIS OF ANABASINE

5.1 Introduction

The number of investigations into the alkaloids of tobacco is legion[103] - in fact, tobacco has been studied more than any other plant. Commercial tobacco is derived from Nicotiana tabacum, while most of the biosynthetic studies have been performed on other Nicotiana species e.g. glutinosa, rustica and glauca.

The main alkaloid in N. glauca (tree tobacco) is anabasine, so called since it was first isolated from Anabasis aphylla, where it is found having the (S)-configuration.[104]



(17)

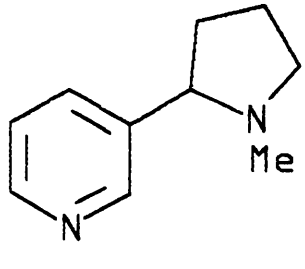
The existence of anabasine (17) has been noted in many plant species of different families.[105] Anabasine from N. glauca is almost racemic but the optical rotations have only occasionally been recorded in other species, and conflicting opinions exist in the literature. Table 18 illustrates a selection of optical rotations of anabasine isolated from different plant species.

Table 18. Sources of Anabasine and its Optical Rotation[105]

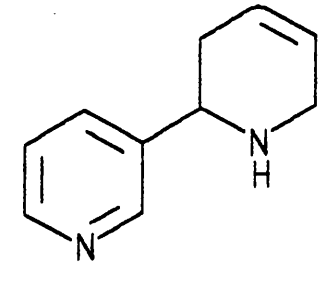
Family	Species	Optical Rotation $[\alpha]_D$
Chenopodiaceae	Anabasis aphylla	-52° to -80°
Solanaceae	Duboisia myoporoides	-0.44°
Chenopodiaceae	Haloxylon salicornicum	-58.1°
Zygophyllaceae	Malococarpus crithmofolius	+10°
Asdepiadaceae	Marsdenia rostrata	0°

Many tracer experiments have been performed on Nicotiana species. There are numerous methods of administration available, and the method used can have a significant bearing on the results.[106] The most practised methods are hydroponics, wick, excised roots and stems, cell-cultures, enzyme systems, stem injection, and leaf administration. Hydroponics was the most commonly used method of administration in the early tracer experiments. In this method, tracer is added to a solution where intact roots are growing; good incorporations can be achieved as roots are the main site of biosynthesis.

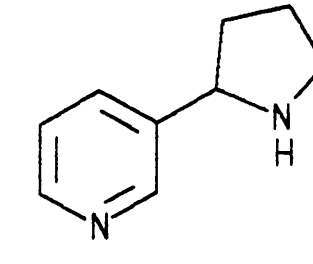
Attempts to elucidate the biosynthetic pathway to the tobacco alkaloids [nicotine (116), anabasine (17), anatabine (117) and nornicotine (118)] have involved feeding of putative precursors.



(116)

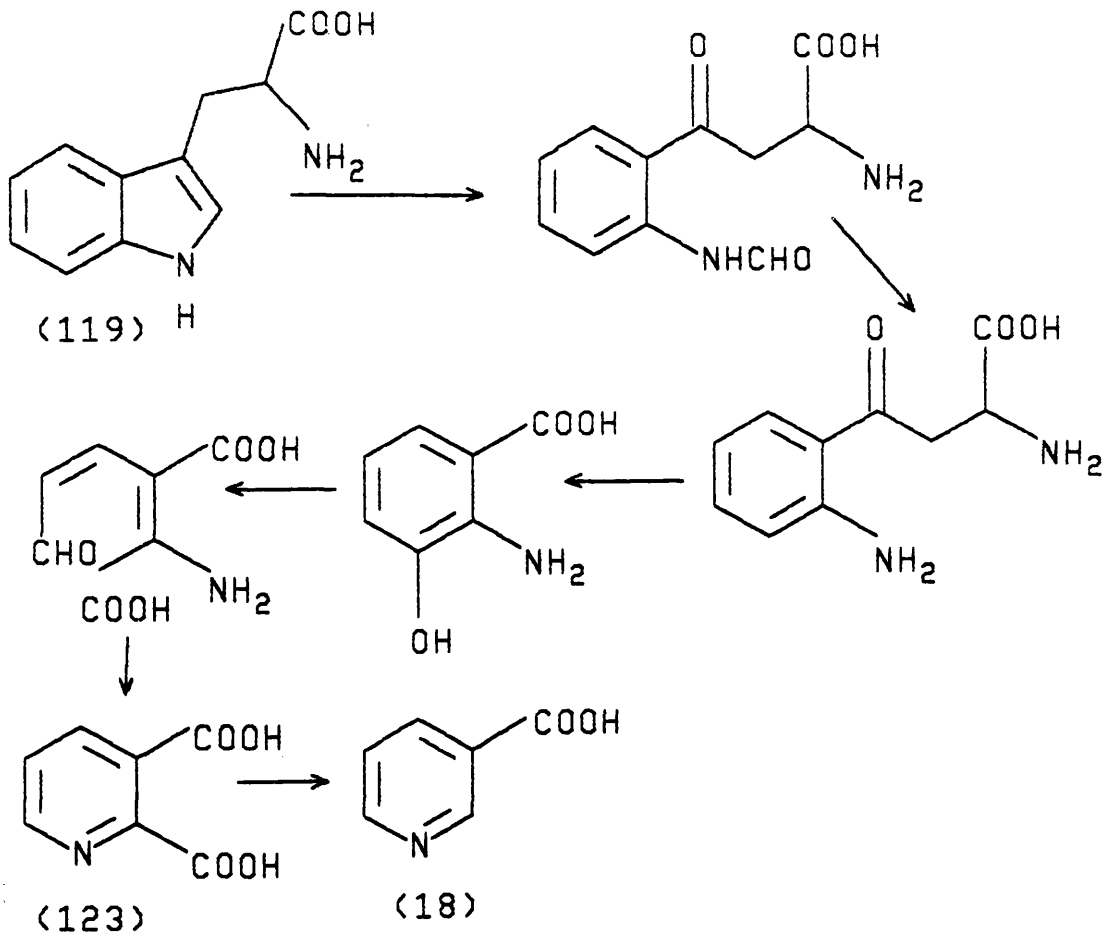


(117)



(118)

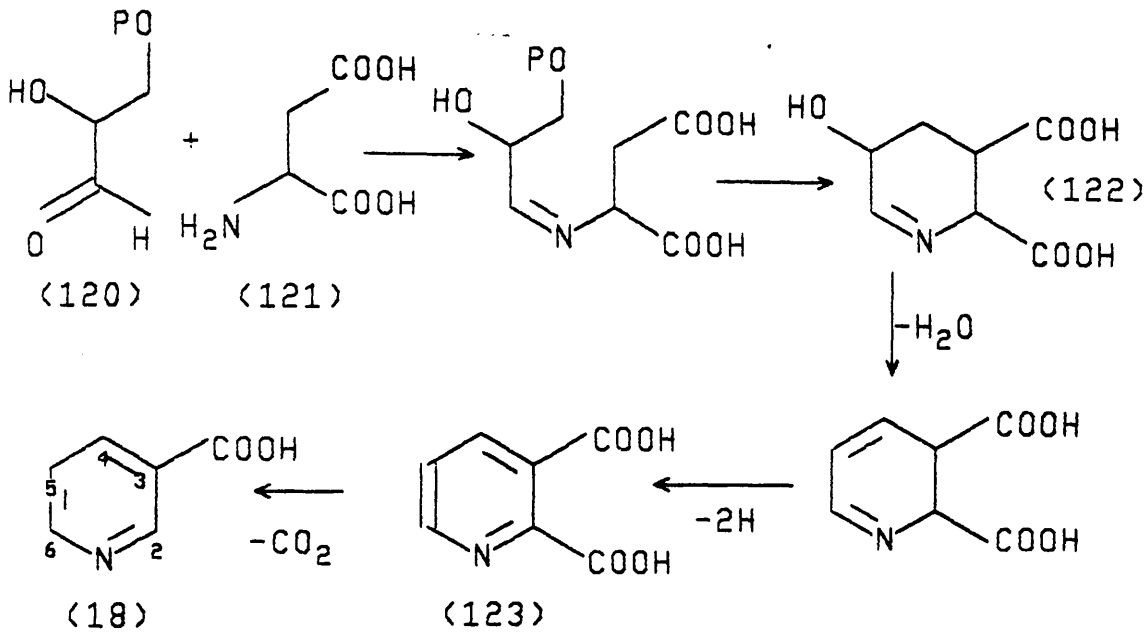
Nicotinic acid (18) was found to be the source of the pyridine ring, both in nicotine (116) and in anabasine (17).[107] The origin of the nicotinic acid was found not be be from tryptophan (119), as it is in micro-organisms and animals (Scheme 44).[108] The results from many feeding experiments suggested a new different pathway - from 3-phospho-D-glyceraldehyde (120) and aspartic acid (121).



Scheme 44

In the proposed pathway the Schiff base cyclises to yield a tetrahydropyridine derivative (122), which undergoes degradation, then oxidation occurs to afford the pyridine ring of quinolinic acid (123). The

quinolinic acid is then decarboxylated to give nicotinic acid, a process which is known to occur in plants (Scheme 45).[109]

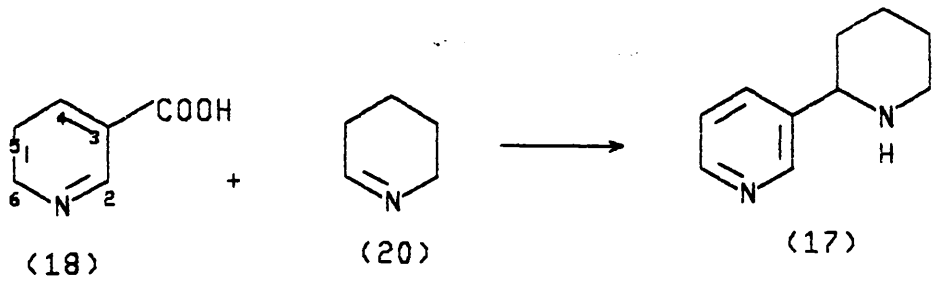


Scheme 45

Numerous feeding experiments have produced results consistent with this pathway, but there remains some doubt about the validity of this route, and there is still much to be learned about the manufacture of nicotinic acid in plants.

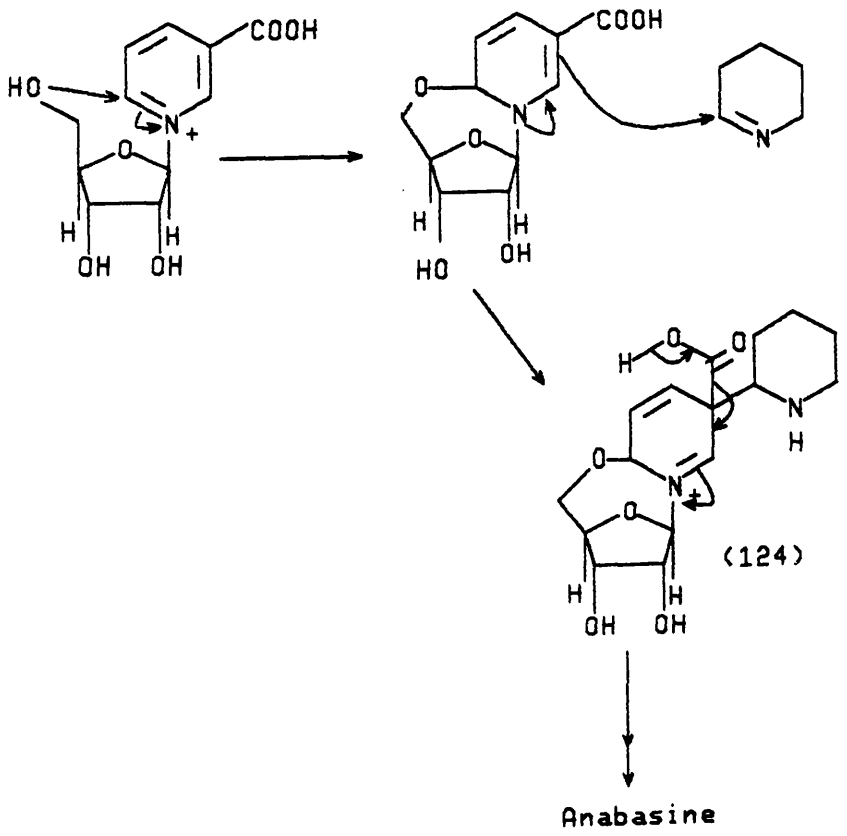
For nicotinic acid to be incorporated into anabasine, attack must occur from C-3 to C-2 of the piperidine ring (Scheme 46). The mechanism of activation is under debate - the formation of anabasine requires an intermediate nucleophile at C-3 for attack at an electron deficient C-2 position of the imine/iminium ion. [The piperidine ring

is thought to be derived from Δ'-piperideine (20)].



Scheme 46

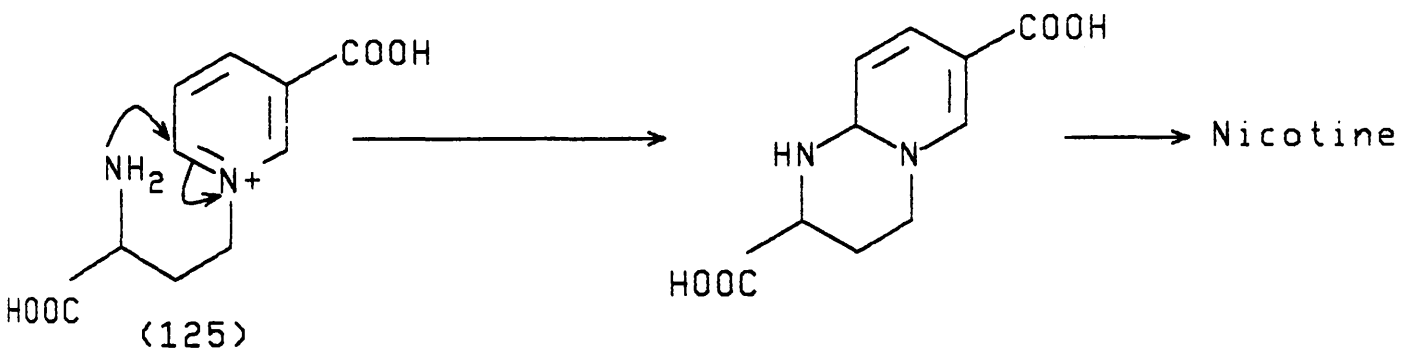
Several mechanisms of activation have been proposed. An N-glycoside, e.g. N-ribosyl derivative, was proposed by Wenkert, which involves a 1,6-dihydropyridine derivative, an enamine, by reaction of an hydroxyl at C-6.[10] The enamine could then attack the piperidine ring, producing compound (124), which on decarboxylation and removal of the carbohydrate residue would yield anabasine (Scheme 47).



Scheme 47

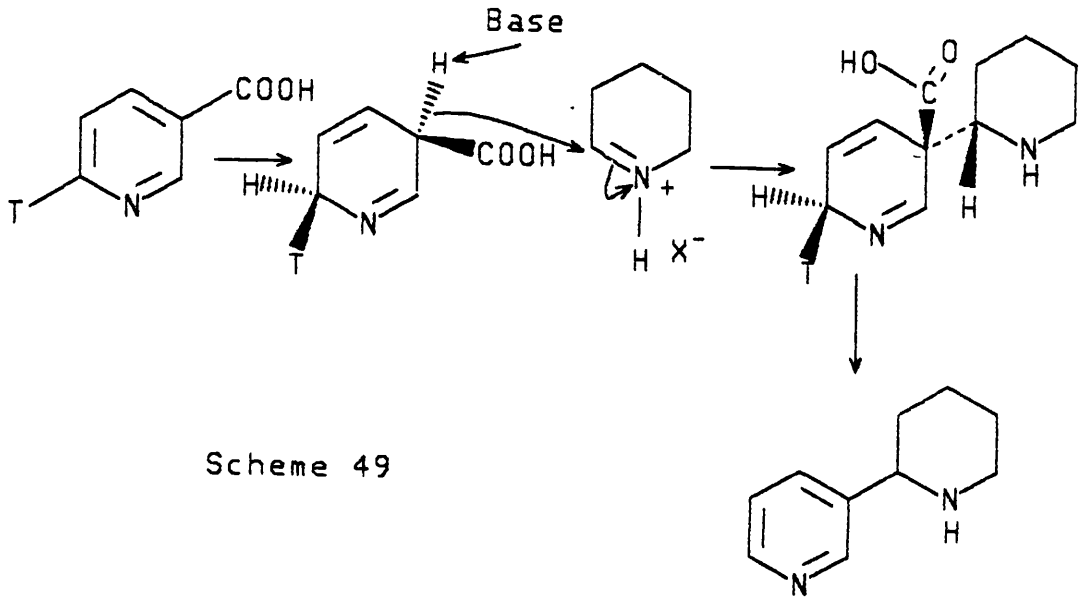
Nicotinic acid N-glycoside has been found in tobacco and is formed from nicotinic acid but it is not a better precursor than nicotinic acid.[111]

Although the pyridine moiety of nicotinamide adenine dinucleotide (NAD) was incorporated into nicotine in N. rustica the efficiency was the same as that of nicotinic acid.[112] Nicotianine (125), found in tobacco leaves, was also proposed as an intermediate, with activation at C-3 in the intermediate, as illustrated in Scheme 48. It was shown, however, to be a much poorer precursor than nicotinic acid.[119]



Scheme 48

Removal of hydrogen at C-6 was found to occur when nicotinic acid is converted into anabasine.[114] After experiments with tritium-labelled nicotinic acid, an alternative mechanism was proposed to account for these observations (Scheme 49).[115] It must be stressed, however, that this mechanism is hypothetical and the method of activation is still unproven.



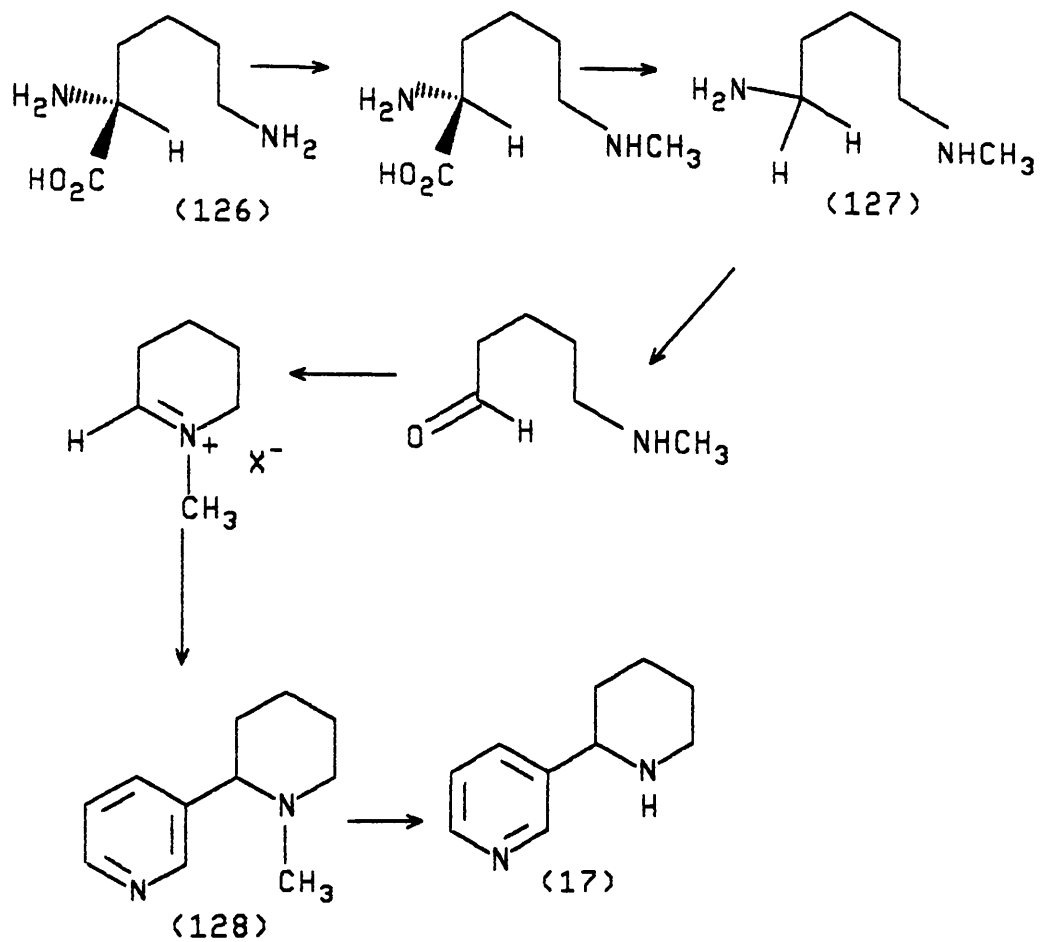
Scheme 49

(17)

The piperidine ring of anabasine is derived from lysine (14). The presence of a symmetrical intermediate was discounted by feeding [2-^{14}C]lysine to N. glauca, when all the radioactivity was found to be located at the C-2' position. Hence free cadaverine is not an intermediate. [1,5-$^{14}C_2$]Cadaverine, however, was incorporated into the piperidine ring of anabasine.[116]

It was proposed that the unsymmetrical incorporation of lysine occurred as a consequence of methylation on the ε-amino group to yield ε-N-methyllysine (126), which is decarboxylated to give N-methylcadaverine (127).[117] Stereospecific oxidation of the primary amino group affords 5-methylaminopentanal, which cyclises then condenses with nicotinic acid to give N'-methylanabasine (128), which could be demethylated to produce anabasine (Scheme 50). This is analogous with the formation of nornicotine (118) from nicotine (116), and Dawson has

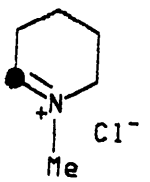
shown that N'-methylanabasine is indeed converted into anabasine in N. glutinosa.[118]



Scheme 50

This hypothesis was tested by feeding [2-^{14}C]-N-methyl-Δ^1-piperideinium chloride (129) to N. tabacum and N. glauca. It was concluded that the incorporation of (129) to produce N'-methylanabasine was an example of an aberrant synthesis from an unnatural precursor. A more recent model proposes a concerted decarboxylation of lysine and subsequent oxidation to δ-aminovaleraldehyde in complexation with

pyridoxal phosphate. In this model, the cadaverine intermediate is predominantly bound and only weakly exchangeable.[119]



(129) ●^{14}C

It has been demonstrated that cadaverine has a very interesting and unique role in anabasine biosynthesis. Cadaverine was shown to stimulate the production of anabasine in hairy root cultures of N. rustica transformed with Agrobacterium rhizogenes. These cultures normally produce mainly nicotine.[120] Furthermore, when unlabelled cadaverine was fed together with L-[U-^{14}C]lysine to N. hesperis, the incorporation of lysine into anabasine was undiminished, but the anabasine production was greatly increased. This suggests that the utilisation of lysine and cadaverine occurs by distinct enzyme systems.

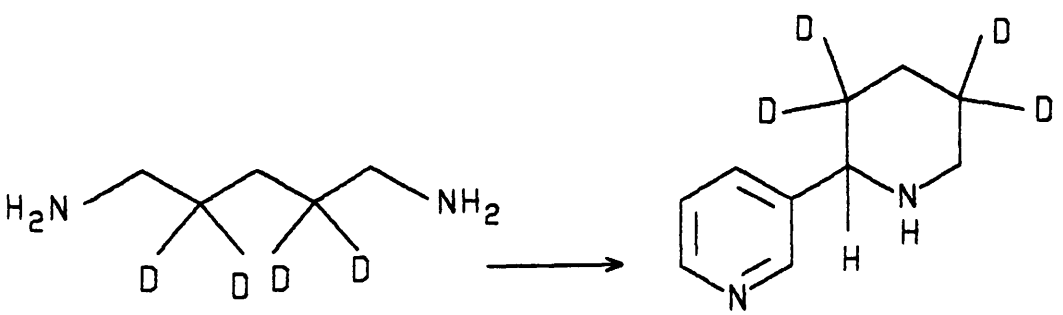
A pathway, which involves cadaverine as the precursor of both rings of anabasine, was proposed in the late 1950s from evidence from pea and lupin extracts in vitro.[121,122] This has never been demonstrated in vivo.

5.2 Investigations into the Biosynthesis of Anabasine

It was decided to feed deuterium-labelled cadaverines to Nicotiana tabacum cultures and use the ^{2}H n.m.r. spectra thereby obtained to determine whether cadaverine could indeed be a precursor for both rings of anabasine. The ^{1}H n.m.r. spectrum of anabasine is

shown in Figure 21. The main features are the peaks at δ 8.58, 8.47, 7.72 and 7.25 p.p.m., corresponding to the aromatic protons on C-2, C-6, C-4 and C-5, respectively. The other important signals are at δ 3.65, 3.20 and 2.80 p.p.m., corresponding to the [illegible] protons on C-2' and the protons on C-6', respectively.

[2,2,4,4-$^2H_4$]Cadaverine dihydrochloride (56) (Chapter 2) was administered by research workers at the A.F.R.C. Food Research Institute to Nicotiana tabacum cultures and a mixture of anabasine, anatabine, nicotine and nornicotine were extracted. Anabasine was separated from the other alkaloids by h.p.l.c. and purified. Clear evidence of almost 100% deuterium incorporation into the 3- and 5-positions of the piperidine ring was obtained from the ^1H n.m.r. spectrum of anabasine due to the absence of signals for C-3 and C-5 protons and changes in coupling at the 2- and 6-positions at δ 3.68 and 2.80 p.p.m. The signal at δ 3.68 p.p.m. had become a singlet, while the peak at δ 2.80 p.p.m. had changed to a doublet. No incorporation of deuterium was observed in the pyridine ring (Scheme 51, Figure 22), so cadaverine was not incorporated into this part of the anabasine molecule.



Scheme 51

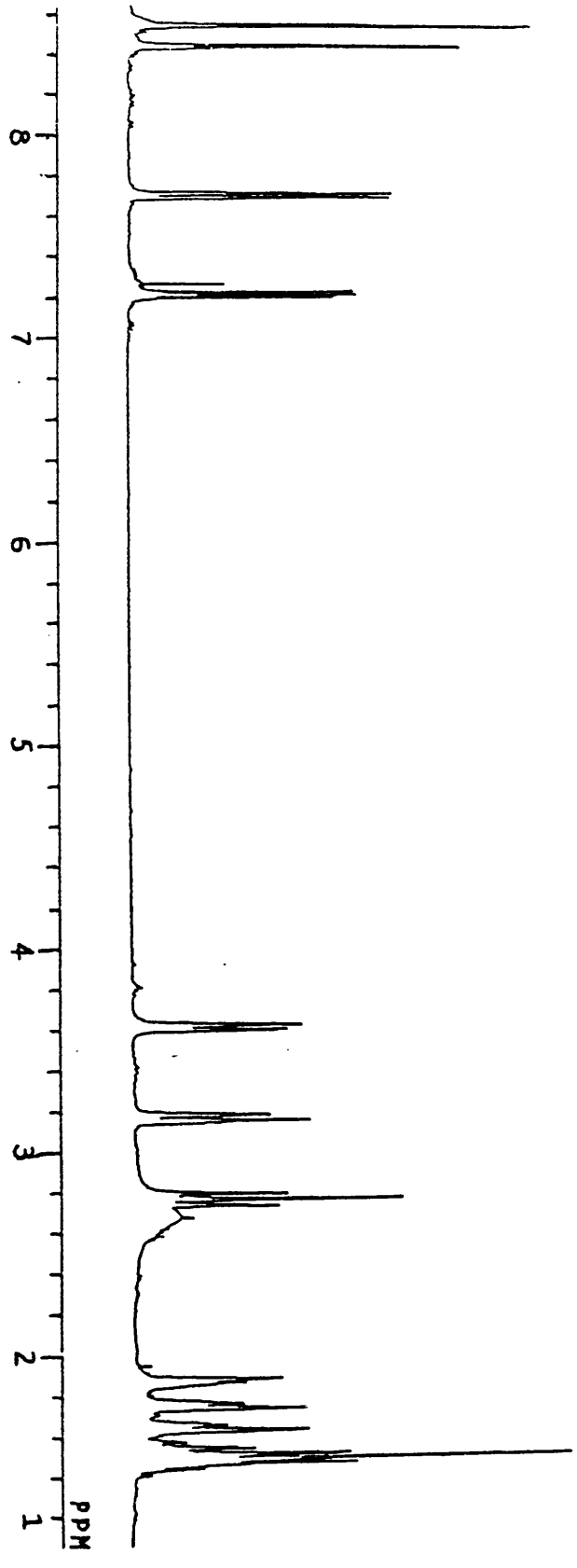
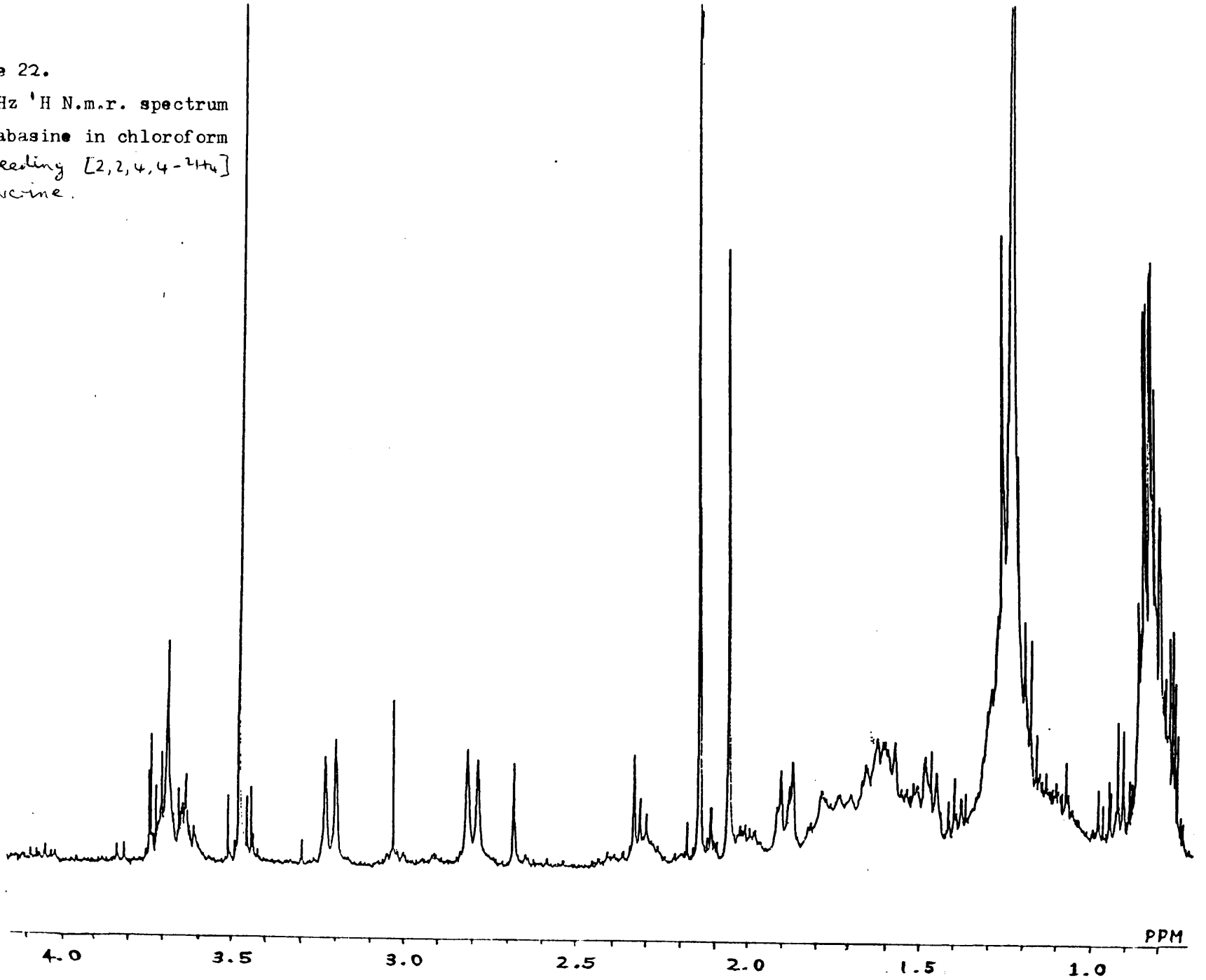


Figure 21. 200 MHz spectrum of anabasine

Figure 22.
200 MHz ^{1}H N.m.r. spectrum of anabasine in chloroform after feeding [2,2,4,4-$^2H_4$] cadaverine.



This evidence indicates that, in vivo, cadaverine is not a precursor for the pyridine ring of anabasine in Nicotiana cultures.

Similarly, (R)- and (S)-[1-^{2}H]cadaverine dihydrochlorides (35) and (36) were fed to Nicotiana root cultures at Norwich. The alkaloidal extracts were again a mixture of nicotine (116), nornicotine (118), anabasine (17) and anatabine (117). The crude samples were submitted for ^{2}H n.m.r. spectroscopy, and the spectra thus obtained are shown in Figures 23 and 24. The deuterium spectra show labelling corresponding to the piperidine ring of anabasine with no signals corresponding to the pyridine portions.

Analysis of the spectrum produced after feeding (S)-[1-^{2}H]-cadaverine shows peaks of equal intensity at δ 3.21 and 2.61 p.p.m., corresponding to the axial and equatorial positions of C-6' of anabasine, by comparison with the ^{1}H n.m.r. spectrum (Figure 21). The three signals in the spectrum from (R)-[1-^{2}H]cadaverine at δ 3.63, 3.21 and 2.61 p.p.m. in the ratio of 2:1:1 correspond to the axial and equatorial positions of C-6' and to the C-2' deuterium. Hence the signals observed in both spectra appear to be due entirely to anabasine, with little or no contribution from the other alkaloids present.

The distribution of ^{2}H signals in the spectrum from (R)-[1-^{2}H]-cadaverine can be explained by the root cultures forming equal amounts of the two enantiomers and two diastereomers of anabasine (Scheme 52). The ^{2}H n.m.r. spectrum observed is a combination of the signals from all four labelled compounds.

Figure 23 and Figure 24. 55.28 MHz ^2H-$\{^1H\}$ N.m.r. spectra of anabasine in chloroform.

(a) sample of anabasine derived from (R)-[1-^2H]-cadaverine.

(b) sample of anabasine derived from (S)-[1-^2H]-cadaverine.

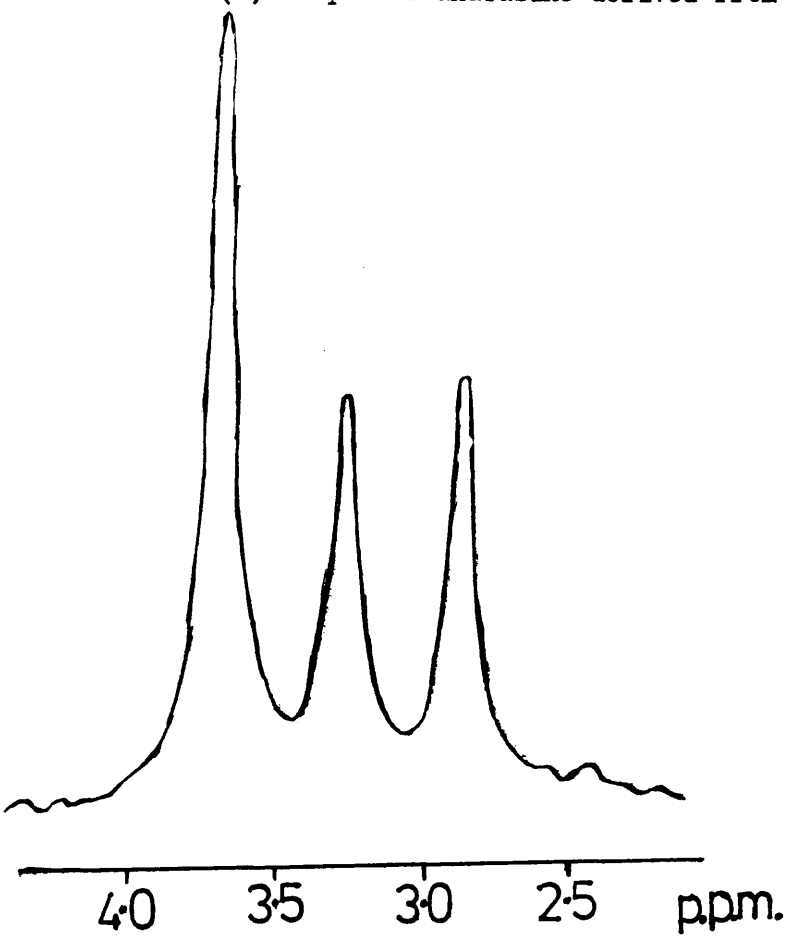


Figure 23

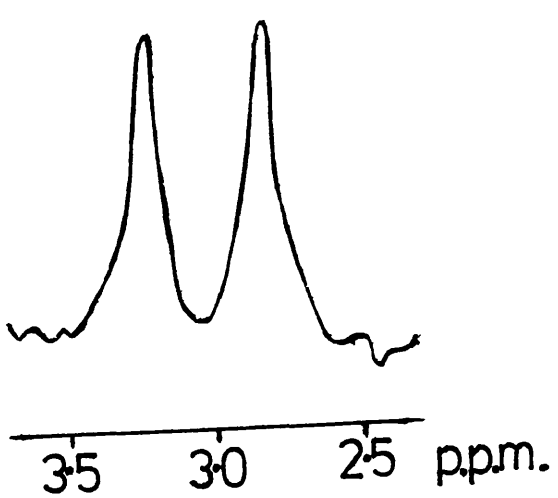
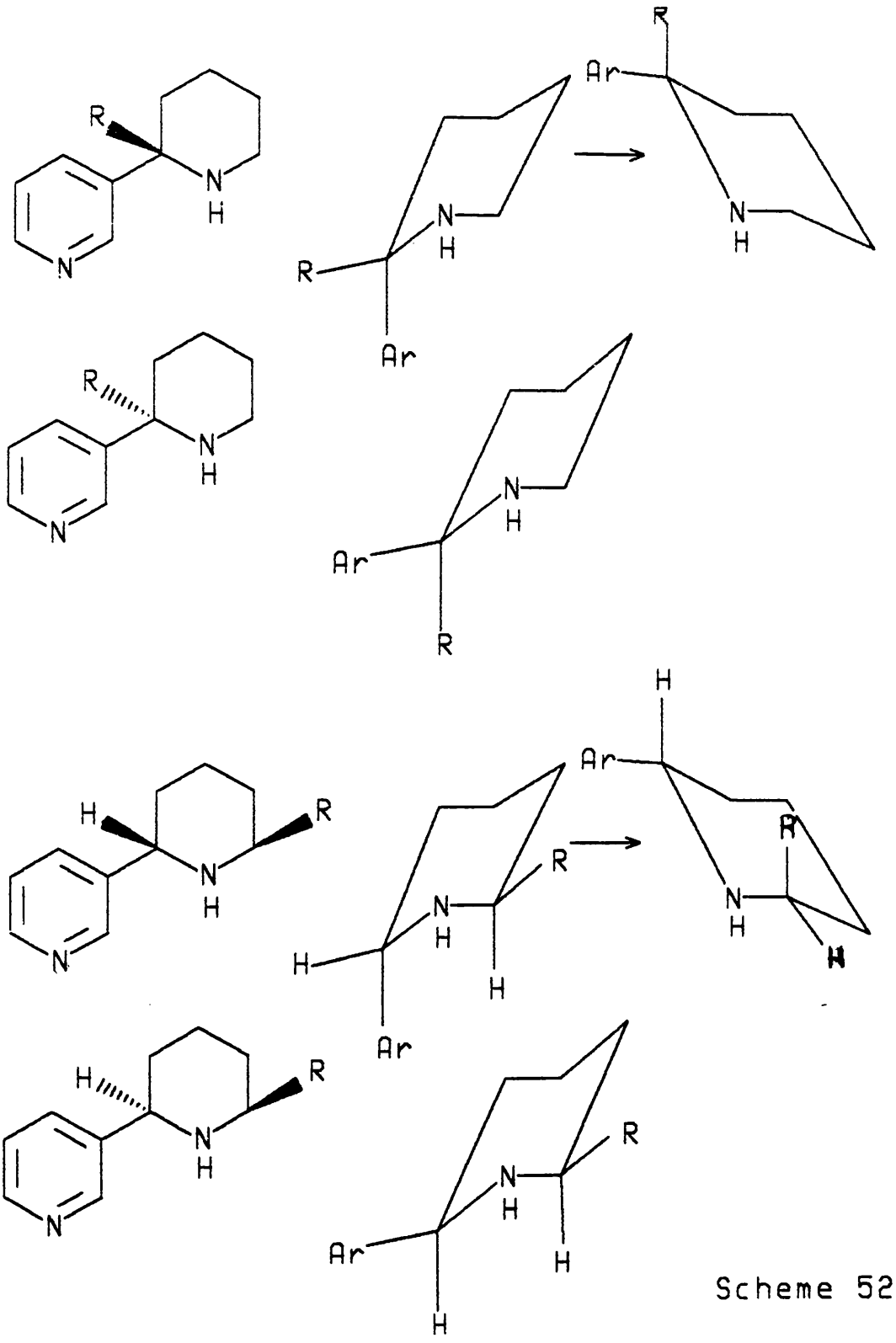


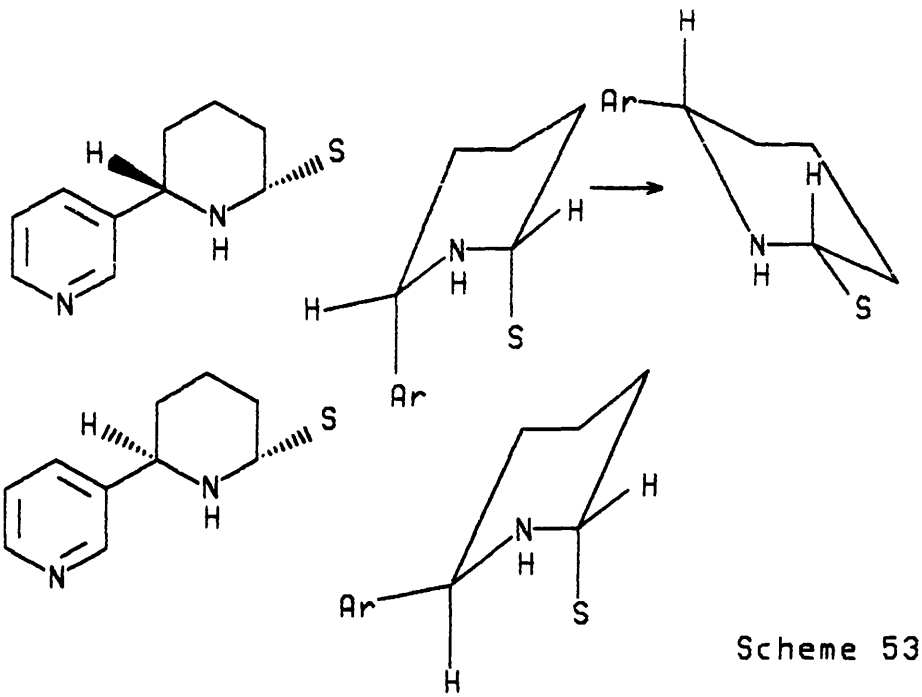
Figure 24



Scheme 52

In the more stable chair conformation, the aromatic group adopts the equatorial position. When the deuterium is present at the 2' position, it is in the axial position. However, when the deuterium becomes incorporated at position 6', the label is equally distributed at the axial and equatorial positions, because of the formation of racemic anabasine.

The argument is similar in the case of the spectrum from (S)-[1-^{2}H]cadaverine (Scheme 53).



Scheme 53

Equal amounts of deuterium will be situated at the axial and equatorial sites of C-6'.

From these results, several conclusions can be drawn about the biosynthesis of anabasine:

(i) Cadaverine is not a precursor for the pyridine ring - there are no ^2H signals in the pyridine region of the spectra. This confirms the earlier result after feeding [2,2,4,4-$^2H_4$]cadaverine dihydrochloride.

(ii) In the conversion of cadaverine into 1-aminopentanal, it is the pro-R hydrogen that is retained, and the pro-S hydrogen that is lost.

(iii) The nicotinic acid must be able to attack the Δ^1-piperideine moiety at C-2' from either the re or si face to produce the structures shown in Schemes 50 and 51. If the process was stereospecific, only one of the 6' sites, axial or equatorial, would be labelled with deuterium.

In order to account for the observations, several proposals can be made:

(i) The process is non-enzymic.

(ii) The process is enzymic, but is not stereospecific.

(iii) A mixture of (i) and (ii).

The integration of the ^2H signals after feeding (R)-[1-^2H]cadaverine to produce labelled anabasine makes it clear that racemisation does not occur after anabasine has been formed. Published work provides evidence that the formation of hygrine from acetoacetic acid and Δ^1-pyrroline can be non-enzymic.

These investigations are being continued by Mr. A.B. Watson with co-operation from the A.F.R.C. Food Research Institute, Norwich (CASE award). It is intended to produce anabasine on a larger scale, after feeding Nicotiana cultures with (R)- and (S)-[1-^2H]cadaverines with the aim of separating anabasine from the other products of the

biosynthesis. More information can then be collated - ^{1}H n.m.r. (loss of H signals and changes in couplings) and m.s. (for ^{2}H content). It is also hoped to extend the studies further by producing analogues of anabasine after feeding derivatives of cadaverine to Nicotiana root cultures.

CHAPTER SIX

SYNTHESIS OF CADAVERINE DERIVATIVES FOR BIOLOGICAL TESTING

6.1 Introduction

Much use has been made of cadaverine analogues in the formation of polymers, in particular, polyureas and polyamides. For example, 3,3-dimethylcadaverine (108) was reacted with hexanedioic acid, 1,4-diamino-2-methylbenzene, 1,18-octadecanediamine and octadecanedioic acid to make a polymer used as an adhesive in the textile industry.[123] The syntheses were carried out under extreme conditions usually at elevated temperatures and pressures.[124]

A known cadaverine derivative, 3,3-dimethylcadaverine, was synthesised under mild conditions in order to investigate the biosynthesis of quinolizidine alkaloids. This work is described in Chapter 3.

In recent years, a great deal of interest has been expressed in the synthesis of polyamines such as cadaverine, putrescine and spermidine and their derivatives.[125] These polyamines are widely distributed in nature[126] and play important roles in various biochemical processes.[127] Compounds which have an inhibitory effect on the biosynthesis of polyamines are potentially antibiotic or chemotherapeutic agents.[128] A series of cadaverine derivatives was prepared in order to determine the relative biological effects of the different derivatives.

The syntheses and subsequent testing of these analogues are discussed in the following sections.

6.2 Synthesis of Cadaverine Derivatives

The series of cadaverine derivatives successfully synthesised is shown below [(130) to (135), (137) and 111)]. Attempts at preparing (136), (142) and (145) proved unsuccessful. The compounds can be divided into three groups, their syntheses being discussed separately:

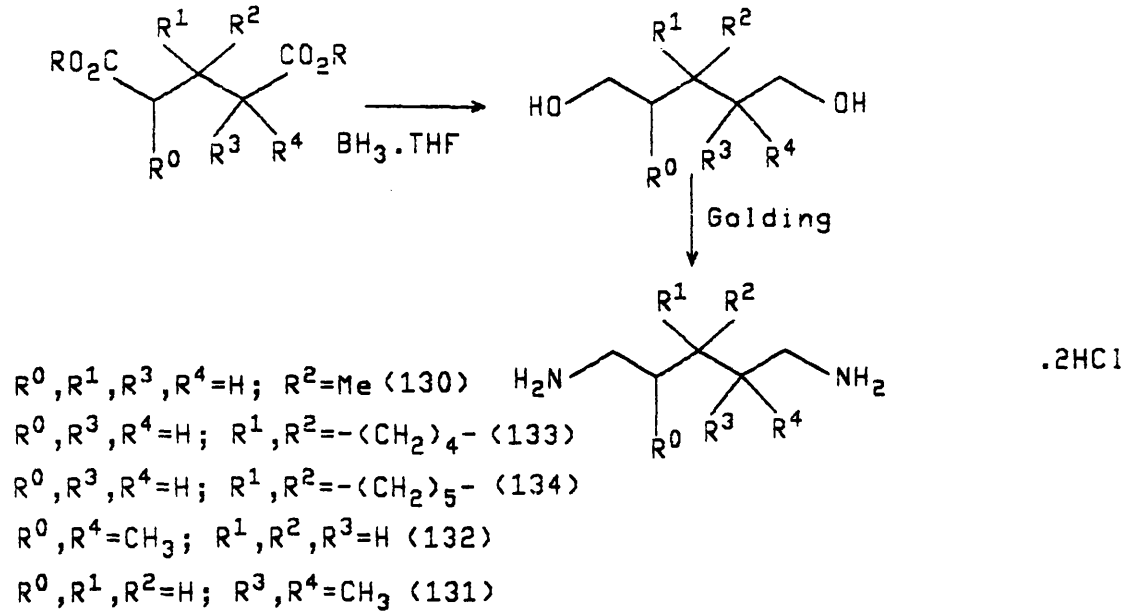
(i) Cadaverine derivatives having only methyl substituents.

(ii) Cadaverine derivatives containing an oxygen atom.

(iii) Cadaverine derivatives containing fluorine atoms.

6.2.1 Synthesis of Cadaverine Derivatives with Methyl Substituents

Preparation of compounds (130) to (134) followed the same general pathway (Scheme 54), utilising the corresponding substituted glutaric acid or anhydride. The acid or anhydride was reduced to the diol using diborane in THF, resulting in yields of around 75%. The reaction could be followed by i.r. spectroscopy since the carbonyl peak of the acid or anhydride disappeared on formation of the diol. The yield for the reduction step was lowered when the starting material was more highly substituted. The diol was then subjected to the "Golding" reaction, as detailed in Chapter 3. The yield of this step varied greatly depending on the substitution pattern of the diol. It was found that when the compound suffered from some steric congestion, as in the cases of 2,2-dimethylpentane-1,5-diol, 3,3-tetra-

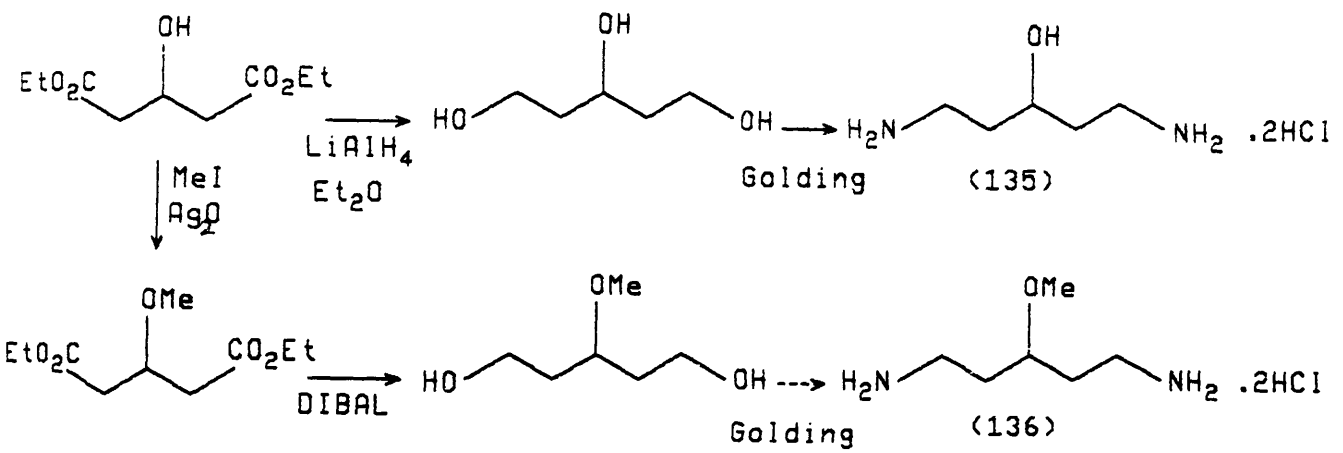


R^0,R^1,R^3,R^4=H; R^2=Me (130)
R^0,R^3,R^4=H; R^1,R^2=-$(CH_2)_4$- (133)
R^0,R^3,R^4=H; R^1,R^2=-$(CH_2)_5$- (134)
R^0,R^4=CH_3; R^1,R^2,R^3=H (132)
R^0,R^1,R^2=H; R^3,R^4=CH_3 (131)

Scheme 54

methylenepentane-1,5-diol and 3,3-pentamethylenepentane-1,5-diol, then the reaction yield for the "Golding" reaction was very low (in the region of 5-10%). The "Golding" reaction is thus useful primarily for straight-chain diols, or those with little substitution. It is not particularly efficient in the reaction of hindered diols. After reacting the sterically hindered diols, the product did not readily crystallise. Often the products remained as gums, and efforts to crystallise them from warm solutions resulted in charring. An attempt at crystallising the cadaverine derivatives with a different counter-ion, sulphate ion, were similarly unsuccessful. In the cases of 3,3-tetra- and 3,3-pentamethylenecadaverine dihydrochloride, different solvent systems for crystallisation were tried, as listed below - all without success.

(i) aq. ethanol/acetone 1:1; (ii) acetone; (iii) water; (iv) n-butanol; (v) isopropanol; (vi) petroleum ether (40/60); (vii) diethyl ether; (viii) ethyl acetate and (ix) cyclohexane.



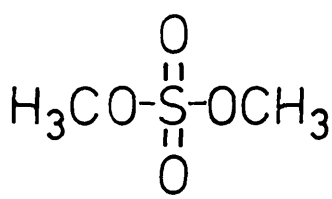
Scheme 55

dihydrochloride. The low yield was probably a result of the highly polar nature of the compound making it difficult to crystallise. There was no evidence of reaction at the secondary alcohol.

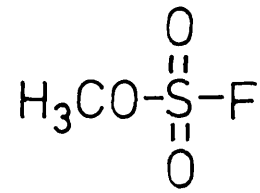
Problems were encountered in the methylation step in the synthesis of 3-methoxycadaverine dihydrochloride (135). Initially, diethyl ether was used as the solvent, and 1.5 molar equivalents of silver (I) oxide and 2.2 molar equivalents of methyl iodide were added to the reaction mixture; then the reaction was heated at reflux for 18h. Work-up of the reaction mixture showed that the reaction product was a mixture of methylated and unmethylated compounds. The reaction was repeated using methyl iodide as the solvent, and the number of

equivalents of silver oxide was increased to two. Again the product extracted was a mixture. Attempts to carry out fractional distillation on the mixture were unsuccessful. The product mixture was remethylated to increase the proportion of desired product and the compounds were separated by column chromatography. The Rf (in $CHC\ell_3$) of the product is 0.76, while that of the starting material is 0.65. The absence of the -OH group in the product was verified by i.r. spectroscopy.

Reduction of the methoxydiester by lithium aluminium hydride, although successful for the corresponding hydroxy compound, did not produce the desired result in this case. The ^1H n.m.r. spectrum of the product was complicated, with a peak at δ 3.73 p.p.m., probably an -OH group, and two peaks at δ 3.50 and 3.40 p.p.m. which presumably correspond to -OMe peaks. There were two sets of peaks at δ 4.18 p.p.m. and 2.60 p.p.m., perhaps signifying the presence of two different diesters. The reaction was performed twice, the outcome being the same in each case. A small scale DIBAL reduction which was subsequently performed resulted in 59% of crude product. Nevertheless, this approach was abandoned on account of the low weight of the resulting impure material. Pressure of time rendered it impossible to try the route again. In order to produce the methoxy analogue in high yield, a more effective methylating agent must be sought - perhaps dimethyl sulphate (139) or methyl fluorosulphonate (140).



(139)

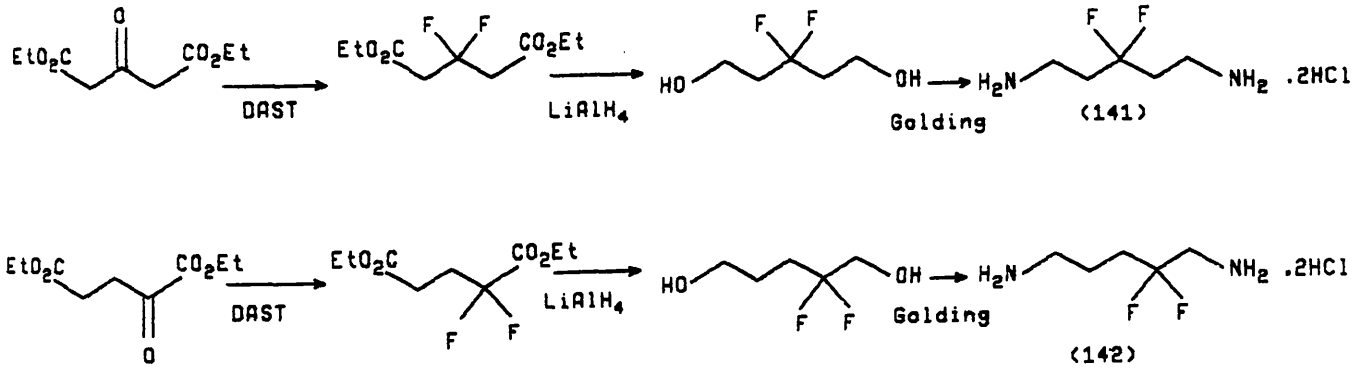


(140)

6.2.3 Cadaverine Analogues Containing Fluorine

Fluorine analogues often have very unusual and interesting effects on biological systems, as detailed in Chapter Two. Fluorinated cadaverine derivatives would be of use both in quinolizidine alkaloid biosynthetic studies and in biological screening. The chosen routes to these compounds follow the same pattern as the previously mentioned analogues. The DAST reagent (diethylaminosulphur trifluoride) was employed to insert the fluorine as required, and the "Golding" reaction was used to form the cadaverine salts.

Details of the route shown in Scheme 39 to 3-fluorocadaverine dihydrochloride (111) have been discussed in Chapter Three. The preferred route to the disubstituted cadaverines from keto-diesters is shown below in Scheme 56.



Scheme 56

Many references exist in the literature for fluorination of ketones using DAST. Usually, the ketones are in isolation - no ester groups are present - however for fluorination of hydroxy compounds the presence of ester groups does not affect the fluorination reaction. Nevertheless, great difficulty was experienced in inserting fluorine atoms into these compounds. The literature references usually employed temperatures of 80°C, with either a non-polar solvent, such as pentane, or no solvent at all. For compound (141), several different conditions were employed and will be discussed in turn:

(i) temperature - 80°C / time - 24h / solvent - pentane;

(ii) temperature - 80°C / time - 28h / solvent - none;

(iii) temperature - 60°C / time - 2h / solvent - none;

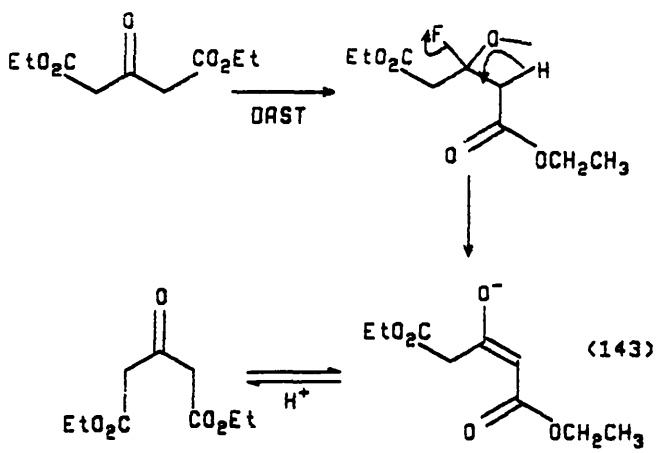
followed by

temperature - r.t. / time - 18h / solvent - none;

(iv) temperature - r.t. / time - 9 days / solvent - none.

Under the conditions shown in (i), it was obvious that the starting material was not miscible with pentane and the starting material turned dark brown after 1h. On work-up, it was apparent that much polymeric material had formed, but no desired product was present. Since the use of pentane as a solvent had been ineffectual, it was decided to perform further reactions without solvent. At 80°C, the reaction mixture charred. The temperature of the next reaction was lowered to 60°C, whereupon a dark orange oil was produced. Following distillation, the orange oil left a black crystalline solid. Spectroscopic data on these samples revealed that neither one was the desired product -

the distillate was impure starting material, and the residue was polymeric material. Finally, the fluorination was performed at room temperature to eliminate charring and reduce formation of polymer. The reaction was followed by t.l.c., which indicated that starting material only was present. Obviously the conditions were not severe enough to bring about reaction. Thus, no conditions were found in which the fluorinated product could be manufactured. The reasons for this remain unclear. Perhaps instability of any initial product formed could influence the reaction. If one fluorine atom was inserted, the relatively labile α-protons could promote the elimination of HF from the molecule to produce the stable conjugated enolate ion (143), which could then be protonated and tautomerise to the starting material (Scheme 57).



Scheme 57

Perhaps at this stage polymerisation would occur. In either case, no product containing fluorine would survive. It is known, however, that in the corresponding reaction with the hydroxy compound, no HF is eliminated. The elevated temperature required for the ketone to react could, however, promote the elimination of HF and the formation of

polymer. Since the other starting material with the ketone alpha to the ester group would not have labile protons next to the inserted fluorine, it was hoped that this fluorination would be more successful.

Again, several sets of conditions were employed:-

(i) temperature - 80°C / time - 18h / solvent - none;

(ii) temperature - 50°C / time - 18h / solvent - none;

(iii) temperature - r.t. / time - 5 days / solvent - none;

(iv) temperature - 80°C / time - 1h / solvent - none.

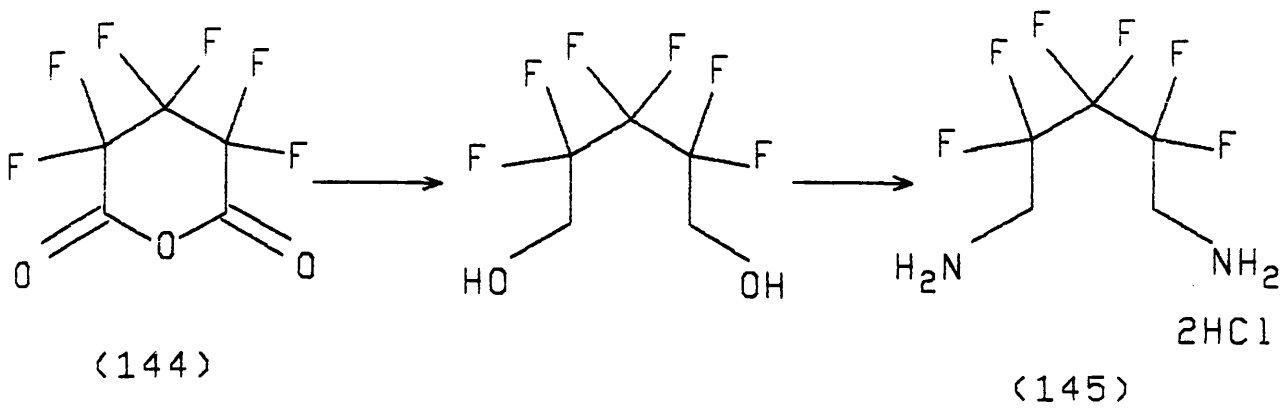
The literature conditions of 80°C for 18h produced a black tar. It was necessary to reduce either the time of reaction or to reduce the temperature. ^{1}H N.m.r. spectroscopy of the tar showed the presence of a multiplet at δ 3.47 p.p.m., possibly due to the protons adjacent to the fluorine atoms. When the reaction was performed at 50°C, aliquots were extracted after 1h and 4h and t.l.c. and ^{1}H n.m.r. data were collected. After 1h, starting material only was present, but after 4h, traces of a product could be observed both on the t.l.c. plate (at Rf value 0.66) and in the ^{1}H n.m.r. spectrum (a small multiplet at δ 3.47 p.p.m.). After the reaction mixture was stirred overnight, the product was a thick dark orange oil, which was difficult to purify. The m.s. trace contained a peak at m/z 226, consistent with it being the desired fluorinated product. There was evidence then that fluorinated product had been found, but unfortunately the purification from polymeric material remained troublesome.

A reduction of temperature co-ordinated with an increase in the length of reaction time was tested next. After the reaction mixture

was stirred for five days at room temperature, it was worked up, but only starting material was recovered. There was no evidence of formation of fluorinated products. From these results, it was clear that an elevated temperature was required to induce the reaction, but extended reaction time resulted in charring of the compounds. The same procedure was tried at 80°C with stirring for 1h. Data from the reaction product revealed that a small amount of fluorinated product was present, but starting material remained. From all the experimental data collected, the most favourable conditions for the fluorination are 80°C for a period of time between 1h and 24h. Further work is needed to establish the optimum time. This would ensure that reaction takes place, but that polymerisation does not occur to any great extent. Due to the lack of time, this work was not continued. If the investigations were to be resumed, studies using a different fluorinating agent would be attractive.

Attempts were made to prepare a fourth fluorine-containing compound, [2,2,3,3,4,4-F_6]cadaverine dihydrochloride (145) from hexafluoroglutaric anhydride (144) by the method shown in Scheme 58.

A model reduction of glutaric anhydride using $LiAlH_4$ was performed in 55% yield. However, attempts to reduce hexafluoroglutaric anhydride by $LiAlH_4$ using cold or reflux conditions did not yield the desired product. Reduction with BH_3.THF resulted in the formation of a diol with no fluorine atoms present. Hence the borane reagent had exchanged the fluorine atoms as well as reducing the anhydride. Again this route was abandoned due to lack of time.



(144) (145)

Scheme 58

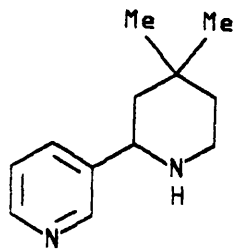
6.3 Testing of the Derivatives Using Biological Screens

The derivatives prepared were tested using several methods for various biological activities.

Anti-fungal assays with Penicillium canadense employing the agar cup method showed that neither 3,3-dimethylcadaverine nor 2-methylcadaverine had any effect on the growth of the fungi. The agar cup method consists of placing solutions of different concentrations of the test substance into wells cut into an agar dish containing growing fungus. The dishes are incubated for 24h and zones of inhibition measured. 3-Methylcadaverine gave a positive result at high concentration (1 mg per cup), while 3-fluorocadaverine inhibited sporing for three days at this concentration. No inhibition of growth was observed of Staphylococcus aureus in anti-bacterial assays using the agar cup method for any of the analogues. These studies were performed by Mrs. P. Tait in the Mycology Unit of the Chemistry Department of Glasgow University. The derivatives are also being tested for anti-fungal activity by Dr. D. Walters at Auchencruive Agricultural College.

Initial tests showed that 3,3-dimethylcadaverine was more effective than α-fluoromethylornithine (a potent inhibitor of ornithine decarboxylase) against plant rusts.

Studies on anabasine (17) with labelled cadaverines had been carried out at the A.F.R.C. Food Research Institute at Norwich by Dr. N. Walton on Nicotiana cultures (Chapter 5). Since high incorporations were attainable, it was decided to feed 3,3-dimethylcadaverine dihydrochloride to Datura stramonium cultures in an attempt to produce an anabasine analogue (146).



(146)

After administration of 3,3-dimethylcadaverine, the tissue culture ceased growing for several months. On replacement with fresh culture medium the culture resumed growth but this growth was abnormal. Callus formation occurred, but the tissue culture never resumed normal growth.

Growth of the Datura culture was completely prevented at a concentration of 2 mM. At this level, 3-methylcadaverine and 2,4-dimethylcadaverine were only partially growth-inhibitory. The results indicate that 3,3-dimethylcadaverine has a very specific toxic action.

Extensive studies using the cadaverine derivatives with diamine oxidase (DAO) enzyme from pig kidney and from pea seedlings

were carried out by Dr. S.K. Ner. The oxidation reaction catalysed by this enzyme is shown in Scheme 58.

$$H_2N\text{-}(CH_2)_n\text{-}CH_2NH_2 + H_2O + O_2 \longrightarrow H_2N(CH_2)_nCHO + H_2O_2 + NH_3$$

Scheme 58.

The experiments were designed to enable a comparison of the oxidation rate of the derivative with that of cadaverine itself. Thus it is possible to estimate how effective a substrate the cadaverine derivative is for DAO enzymes. The derivatives showed a range of oxidation rates and it was found that the two enzymes behaved differently towards the same compound. Several interesting observations were made:-

(i) 3,3-Dimethylcadaverine was a very poor substrate for both enzymes while 3-hydroxy-3-methylcadaverine was oxidised efficiently by both enzymes.

(ii) meso-2,4-Dimethylcadaverine was a poor substrate for both enzymes.

(iii) 3-Methylcadaverine was oxidised efficiently by the pea seedling DAO but not by the pig kidney DAO.

(iv) 2,2-Dimethylcadaverine was a poor substrate for pea seedling DAO but a good substrate for pig kidney DAO.

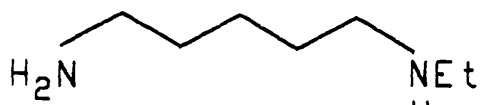
The difference in the results of the two enzymes are difficult to interpret. They indicate, however, that much is yet to be learned about

the active sites of the two enzymes. Further work is in progress in this area.

Inhibition studies on the DAO enzymes were also undertaken by Dr. Ner. Surprisingly, 3,3-dimethylcadaverine was found to have only a modest inhibitory activity, while 2,2-dimethylcadaverine was a good inhibitor of pea seedling DAO. This suggests that the action of 3,3-dimethylcadaverine is against a different enzyme. Obviously, more investigations must be carried out before definite conclusions can be drawn.

Future work could include:-

(i) The synthesis and testing of N-alkyl cadaverines, such as N-ethylcadaverine (147). (Some N-alkyl polyamines have anti-tumour activity).



(147)

(ii) The manufacture of N-acylcadaverines, perhaps using an enzymic route as detailed in Chapter 7.

(iii) The preparation and testing of optically active cadaverine derivatives, to discover if the inhibitory effect is dependent on the stereochemistry of the molecule. Optically active cadaverines may be available by treatment of (±)-diamines with DAO if one enantiomer reacts preferentially.

(iv) More assays should be carried out in order to assist in the evaluation of the derivatives produced.

Routes to a range of substituted cadaverines have been established. The results from the biological screens indicate that some cadaverine derivatives have very interesting properties. More work is clearly required if a compound is to be developed with antibiotic or anti-cancer activity, but the foundation has been laid for continuing work in this area.

CHAPTER SEVEN

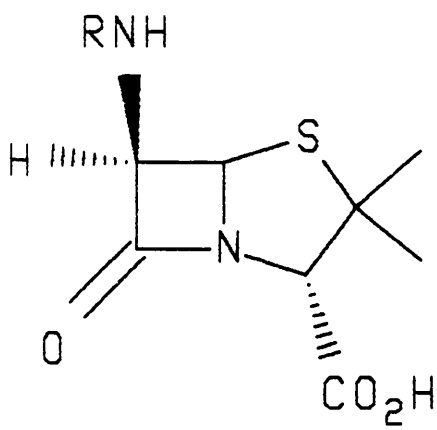
MONOACYLATION OF DIAMINES CATALYSED BY LIPASE ENZYMES

7.1 Introduction

Although enzyme behaviour has been observed for thousands of years, the commercial potential of enzymes as catalysts for chemical reactions has only recently been recognised and begun to be fulfilled.[129,130] Several hundred enzymes are now available from companies such as Aldrich, Boehringer, and Sigma. Enzymes are exceptional catalysts. They are very versatile, catalysing a broad spectrum of reactions usually at room temperature and in aqueous solutions at neutral pH. Furthermore, enzymes are extremely efficient and may increase the rate of reaction by up to 10^{12} times. Of most importance for some applications is the fact that they are very selective in terms of the structure and stereochemistry of their substrates.

A minor, but greatly increasing, role is being played by enzymes in the chemical industry. The best known biotransformation is the manufacture of ethanol from sugar solutions by yeast. Other important developments were the production of acetone and glycerol from starch, both achieved during the first World War. In the 1920s glucose was converted into citrate, which was subsequently transformed (in the 1950s) into glutamate and lysine (14). Currently, the two largest markets for biotransformations are Vitamin C and chemically

modified penicillins. The production of Vitamin C involves an enzymatic step using Acetobacter suboxydons micro-organism for the oxidation of sorbitol into sorbose.[131] Penicillin is produced by fermentation to form Penicillin-G (148) or Penicillin-V (149). These are themselves antibacterial agents, but to improve the biological activity, the side-chain must be changed. This is achieved by removal of the original side chain to give 6-aminopenicillanic acid (6-APA) (150), which can then be chemically reacylated to produce a compound with enhanced bacteriocidal activity, e.g. ampicillin (151).



(1) R=$PhCH_2CO$ (148)

(2) R=$PhOCH_2CO$ (149)

(3) R=H (150)

(4) R=$PhCH(NH_2)CO$ (151)

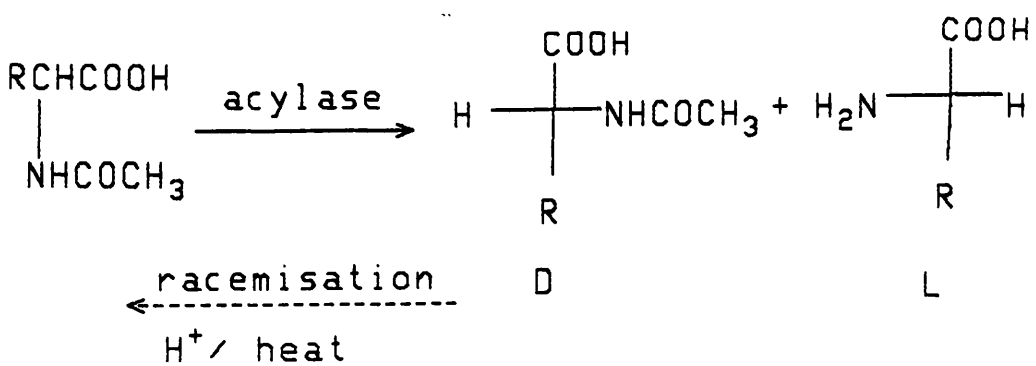
The deacylation can be achieved by either chemical (PCl_5) or biochemical means (by using an acylase from Bacillus megaterium, Escherichia coli or Achromabacter sp.) - both are used in industry. The subsequent modifications are performed using acid chlorides. This example illustrates how chemical and biochemical techniques may be combined in the pursuit of a synthetic goal.[132]

7.1.2 Enzyme Specificity

The synthetic utility of enzymes arises from their ability to discriminate between structural and stereochemical features of their substrates. The most useful enzyme will, therefore, accept a wide range of substrates but will still retain the ability to act stereospecifically on each.

The discrimination of enantiomers by enzymes is well-documented.[133] When an enzyme is enantiomerically specific, transformations of a racemate stop at 50%, the point at which all of one enantiomer has reacted. Often the enantiomer with an unwanted absolute configuration must be discarded, limiting the total yield of useful material to 50%. This reduced yield is often unacceptable, and only where the undesired enantiomer can be recycled is the process synthetically attractive.

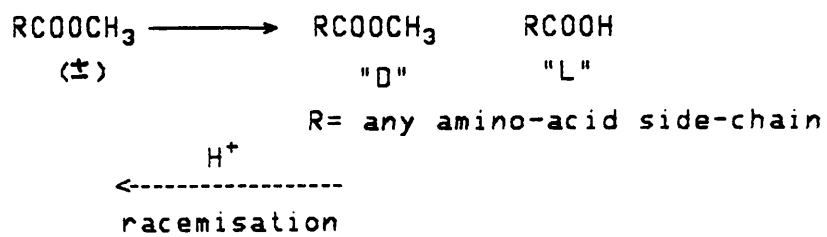
The first major application of enzymes to enantiomer resolutions was the hog kidney acylase-catalyzed resolution of N-acylamino acids.[134] The acylase is stereospecific for L-enantiomers, with the unhydrolysed N-acyl-D-amino-acids being recycled via a chemically induced racemization (Scheme 59).



Scheme 59

Acylase-mediated resolutions of this type have industrial validity to this date.[135] Amino-acids can also be resolved *via* stereospecific hydrolyses of their ester derivatives. Many serine proteases, *e.g.* chymotrypsin, trypsin and subtilisin, exhibit L-enantiomer preference.[136]

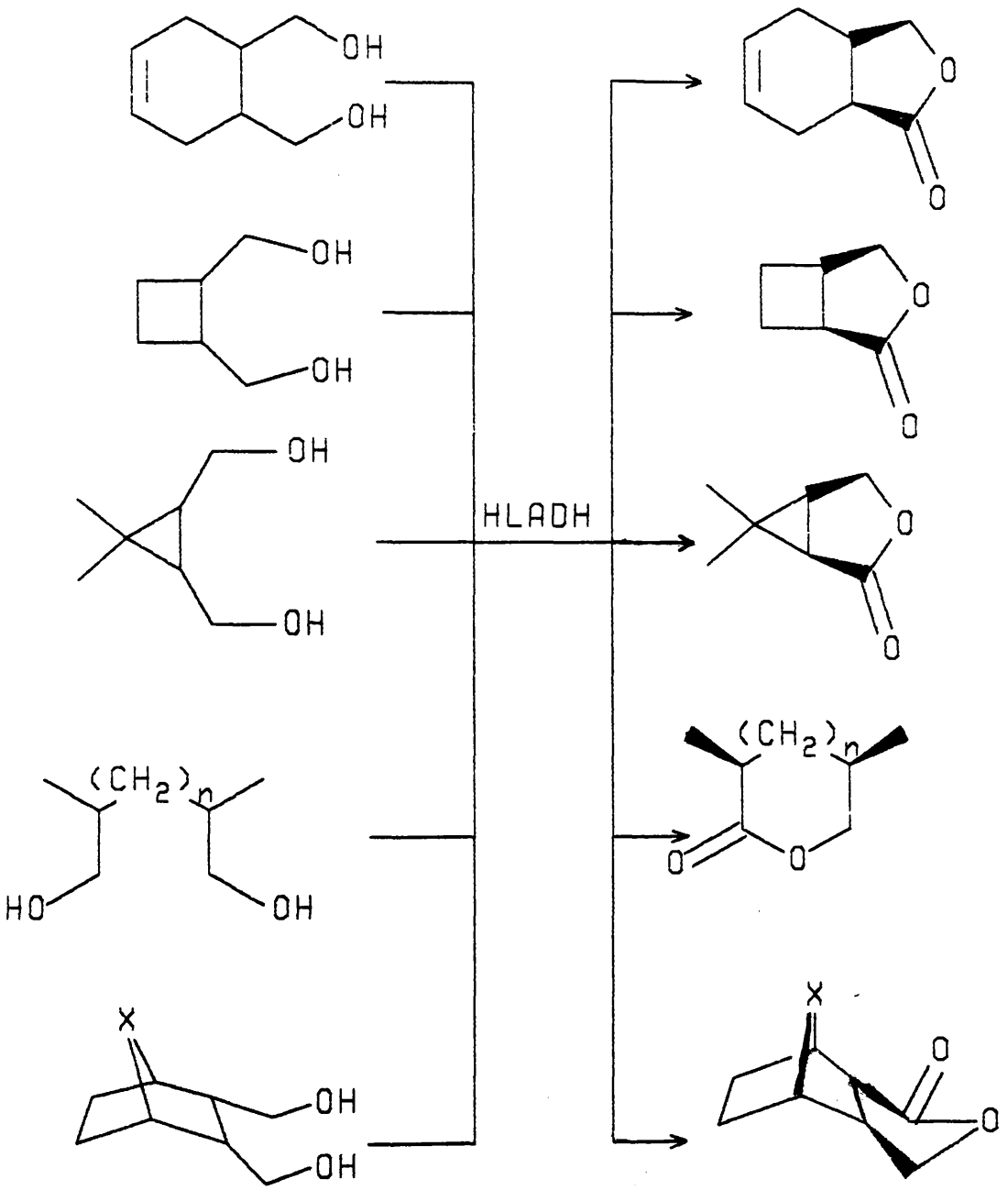
The use of enzymes to resolve racemates is by no means restricted to amino acids. A great deal of work has been performed on racemic esters. Chymotrypsin operates on a broad range of substrates, always showing the same stereospecificity for L-amino-acids. Unreactive enantiomers may be recycled *via* chemical racemization (Scheme 60).



Scheme 60

In asymmetric synthesis, involvement of racemic intermediates is avoided whenever possible. Even when the undesired enantiomer can be recycled, the additional steps involved render the process less favourable. These difficulties can be avoided by exploiting the prochiral stereospecificity of enzymes. In these cases, 100% of a symmetrical substrate may be converted into the desired optically active product.

A broad structural range of meso-diols can be stereospecifically oxidised using horse liver alcohol dehydrogenase (HLADH) (Scheme 61).[137]



Scheme 61[137]

The value of enzymes in asymmetric syntheses is more widely appreciated by organic chemists nowadays, with syntheses incorporating enzymatic or microbiological steps becoming more commonplace. The enzymatic step usually has as its purpose the production of a homochiral compound. The use of enzymes as catalysts in chemical reactions looks set to continue and expand.

7.1.3 Lipases

Lipases are considered to be one of the most versatile groups of enzymes for chemical conversions. In nature, lipases act as catalysts for the hydrolysis of triglycerides in an aqueous emulsion. They work in environments with low water content (at the oil-globule/water interface) and will function in many organic solvents to catalyze the reverse reaction i.e. ester synthesis. Lipases are attractive as catalysts for many reasons - they have low energy requirements, high regio- and stereoselectivity, are non-corrosive, and do not require high temperatures or pressures or expensive co-factors. Lipases are available from animals (the main source being the pancreas), micro-organisms, and plants (e.g wheat germ). The greatest variety of bulk lipases occurs in micro-organisms. Table 19 shows those currently available along with their applications.

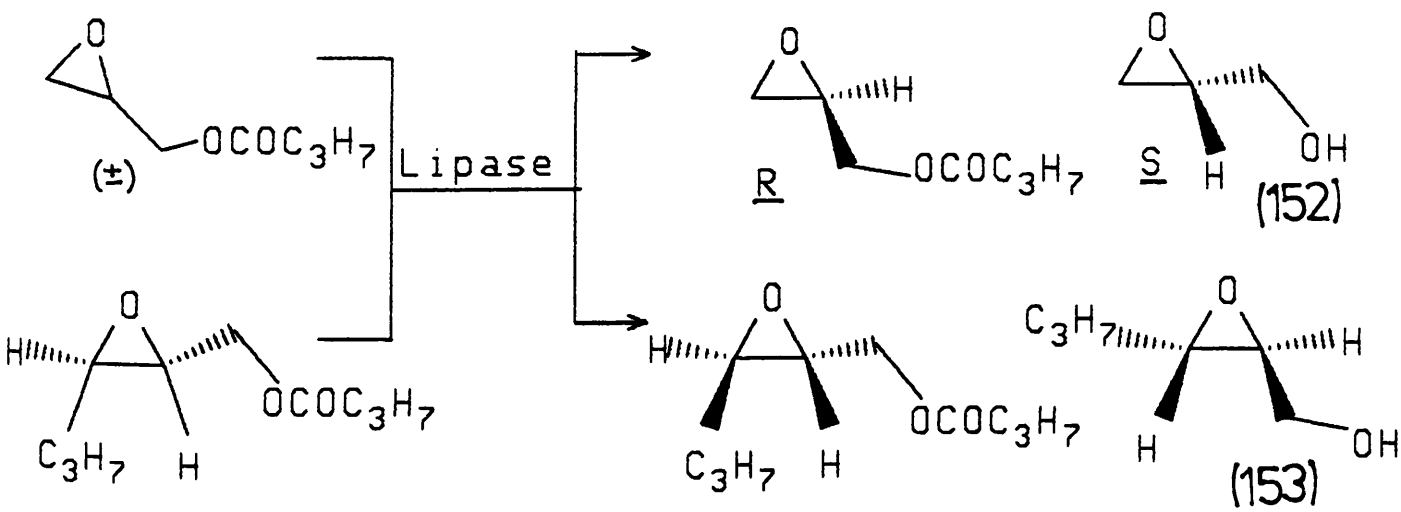
Racemic mixtures can be kinetically resolved by the use of lipases. Many widely available drugs are racemates, with the enantiomers having different pharmacological properties. In the near future, pharmaceuticals will probably have to be single isomers, unless the two isomers have the same properties. For new pharmaceuticals, an

Table 19[130]

Microbial Source	Current Applications/Characteristics
Alcaligenes sp.	Asymmetric hydrolysis of symmetrical esters
Candida cylindracea (rugosa)	Resolution of D,L-chloropropionic acid esters; flavour ester synthesis; effective for primary and secondary alcohols
Geotrichum candidum	High specificity towards C_{18} unsaturated fatty acids
Mucor miehei	Cocoa butter equivalent manufactured by transesterification; ester synthesis
Penicillium cyclopium	Monoglyceride synthesis; also exhibits stereo-selectivity
Pseudomonas fluorescens	Asymmetric hydrolysis of symmetric esters
Rhizopus arrhizus	Esterification
Rhizopus japonicus	Transesterification

asymmetric synthesis will be used to introduce a chiral centre. However, for drugs already on the market, the route may be fixed by the licence and a resolution will have to be performed, either on the final product or on a near intermediate. If the compound has an acid or alcohol moiety nearby, then lipases are ideal for the resolution. An ester could be synthesised chemically, and the lipase should preferentially cleave one enantiomer to give a single optical isomer. Many studies have been made into the resolution of racemic alcohols and carboxylic

acids, using lipases, through asymmetric hydrolyses of the corresponding esters. Yeast (Candida cylindracea) and porcine pancreatic lipase (PPL) are ideal for practical transformations because they are commercially available and relatively inexpensive, require no co-factors for their action, and have a broad substrate specificity. An example of this can be found with chiral epoxides, which are useful synthetic intermediates. Epoxy alcohols such as (152) and (153) have not been readily available, despite the success of the Sharpless epoxidation reaction.[138] The use of PPL-catalyzed hydrolysis of racemic epoxy esters has solved this problem.[139] The hydrolyses proceed with very high enantiomeric specificity for a range of substitution patterns, and enantiomeric excesses (ee) of more than 90% can be achieved for both the epoxy esters and the epoxy alcohols. In this case, the undesired enantiomer is discarded since the starting materials are inexpensive (Scheme 62).



Scheme 62

As has already been mentioned, lipases can operate under virtually anhydrous conditions to catalyze the reverse mode of hydrolysis-ester synthesis. Both reaction modes are reversibly catalyzed by these enzymes (Equation 1).

$$\text{Acyl-OR} + H_2O \underset{\text{ESTERIFICATION}}{\overset{\text{HYDROLYSIS}}{\rightleftharpoons}} \text{Acyl-OH} + \text{R-OH}$$

Equation 1

Klibanov and co-workers found that C. cylindracea converted a carboxylic acid and an alcohol almost quantitatively into the ester in organic solvents.[140] Furthermore, when a racemic acid was used, the reaction was highly stereoselective (Scheme 63).

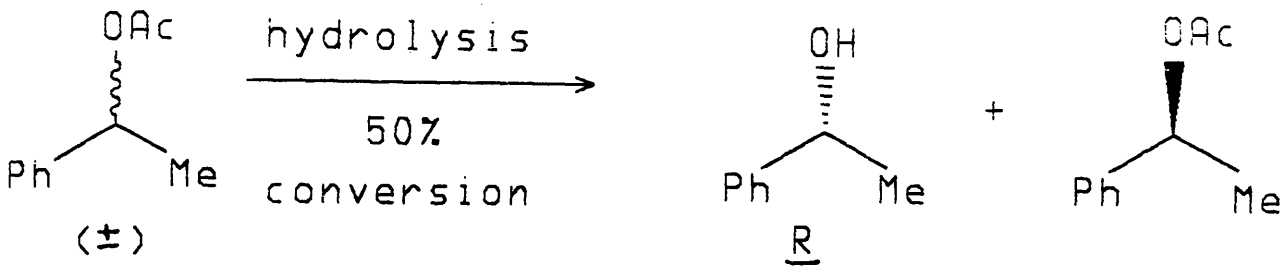
$$R'CHXCOOH + R'OH \xrightarrow{\text{LIPASE}} (R)\text{-}R'CHXCOOR' + (S)\text{-}R'CHXCOOH$$

$R' = CH_3$	X = Br	ROH = n-butyl alcohol	ee(%) = 96
CH_3	Cl	"	95
$CH_3(CH_2)_3$	Br	"	99
Ph	Cl	"	99
Ph	Br	"	79

[140]

Scheme 63

Schneider et al. showed that in aqueous environment the hydrolytic reaction mode is strongly favoured. They prepared alcohols with high enantiomeric purities by hydrolysis of their racemic acetates using an ester hydrolase from Pseudomonas sp. (Scheme 64).[141] They found that the ester synthesis mode was unsuccessful when conventional methods were used - i.e. when racemic alcohols are



Scheme 64

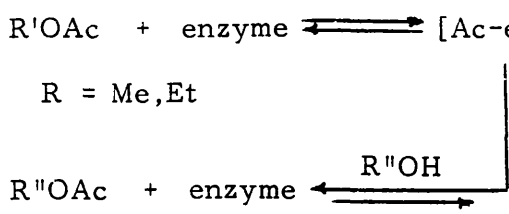
enzymatically converted in an ester matrix usually acting both as an acyl donor and solvent (described below).

$$\text{Acyl-OR}' + \text{enzyme} \rightleftharpoons [\text{Acyl-enzyme}] + \text{R}'\text{OH} \qquad \text{(a)}$$

$$[\text{Acyl-enzyme}] + \text{R}''\text{OH} \rightleftharpoons \text{Acyl-OR}'' + \text{enzyme} \qquad \text{(b)}$$

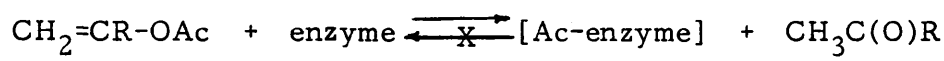
Equation 2

It is known that the intermediate acyl-enzyme complex can be transferred onto other nucleophiles such as alcohols (Equation 2). Since no water is involved, these acyl transfer reactions [in contrast to direct esterifications (Equation 1)] provide ideal conditions for ester synthesis. However, Schneider et al. found that methyl and ethyl acetates were ineffective - transformations were either slow or not observed at all. The alcohols (methanol or ethanol) liberated from the ester matrix could compete with the substrates for the electrophilic acyl-enzyme (Equation 3).



Equation 3

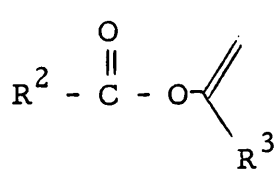
Hence, only if an irreversible route to the acyl-enzymes can be used will these ester syntheses become synthetically attractive. Enol acetates are attractive acyl donors, since the liberated enols are released as acetaldehyde or acetone (Equation 4).[142]



R = H, CH_3 Equation 4

Indeed, Schneider found great rate enhancements in acyl transfer reactions using vinyl acetate as acyl donor.

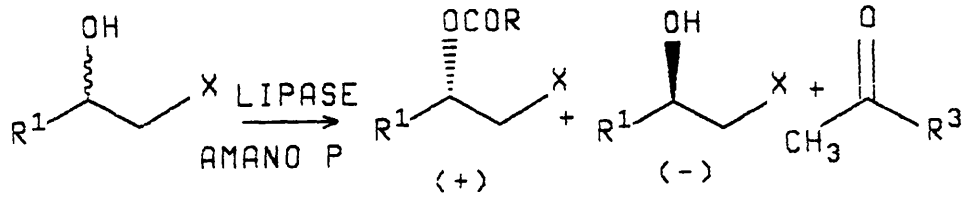
Oda and co-workers found that a lipase, Amano P, from Pseudomonas fluorescens was an excellent catalyst for transesterification of alcohols with the enol esters (154(a)-(d)), allowing rapid and irreversible acylation of alcohols under mild conditions.[143] The



	R_2	R_3
(a)	CH_3	H
(b)	CH_3	CH_3
(c)	n-C_3H_7	H
(d)	n-C_7H_{15}	H

(154)

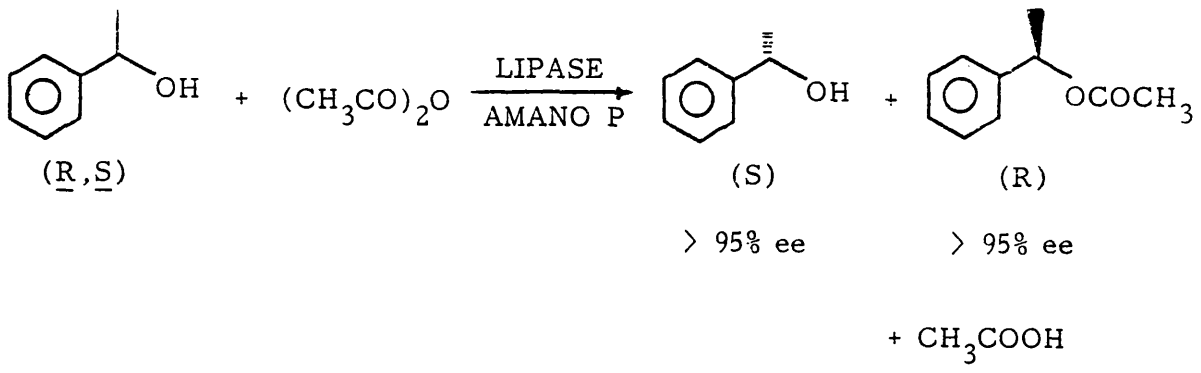
resolution of racemic 2-halo-1-arylethanols was achieved with almost complete stereoselection using this enzymatic reaction. Optically active 2-halo-1-arylethanols are important as synthetic intermediates for compounds of pharmaceutical interest (Scheme 65).[144]



R^1	X	R^2
C_6H_5	Cl	CH_3
2-$C_{10}H_7$	Br	CH_3
4-BrC_6H_4	Br	CH_3
4-$CH_3OC_6H_4$	Br	CH_3
C_6H_5	Cl	n-C_3H_7

Scheme 65

One of the drawbacks of using enol-esters for transesterifications is that the aldehydes formed can give rise to side reactions and can thus decrease the enzyme activity. Cesti and co-workers overcame this particular problem by using various anhydrides as the acylating agent.[145] This reaction does not cause the formation of water or alcohol and the equilibrium is, therefore, completely shifted towards the products because the reverse reaction is thermodynamically unfavoured (Scheme 66).



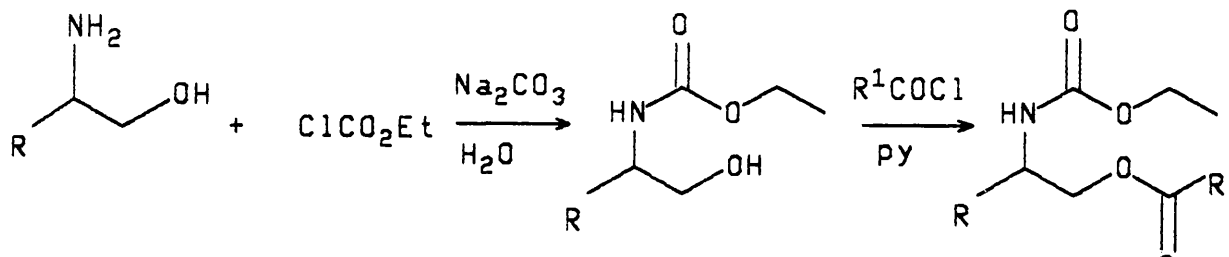
Scheme 66

The choice of enzyme is critical in these reactions. In the above example where anhydrides were utilised, PPL and lipase from C. cylindracea displayed low activity towards secondary alcohols; yet both enzymes showed high activity towards the primary alcohols but poor enantioselectivity. For instance, when 2-phenyl-1-propanol and acetic anhydride were allowed to react in benzene in the presence of PPL immobilised on Celite, the ee of unreacted alcohol was 59% at 50% conversion.

The racemic compounds discussed previously are monofunctional. The selective monoprotection of a given function in a multi-functional molecule constitutes a challenging task in organic synthesis. Recently, lipases and proteases have been successfully used for selective mono-acylation of diols[146] and sugars[147] in organic solvents. Chiral amino-alcohols are of pharmaceutical interest, and they have been under study by several research groups.

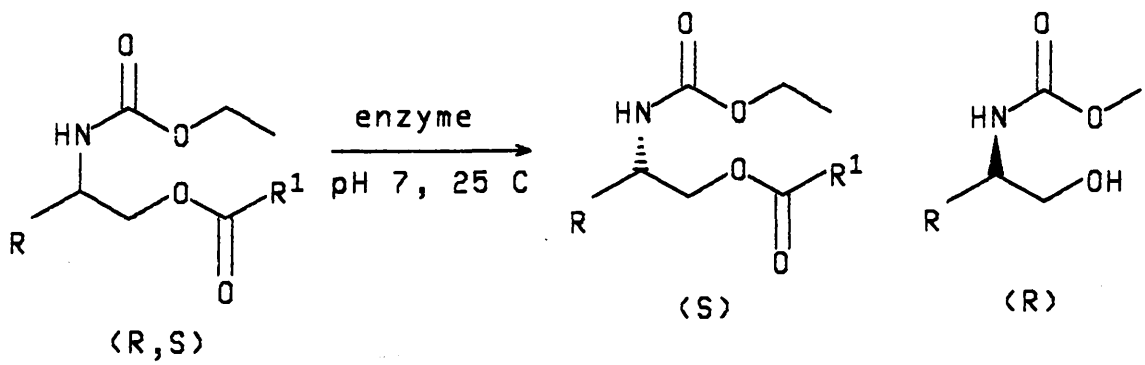
Francalanci and co-workers[148] converted 2-amino-1-alcohols to their N-alkoxycarbonyl derivatives (Scheme 67) and found that their optical resolution was easily obtained by lipase-catalyzed hydrolysis of

carboxylic esters and by lipase-catalyzed transesterification in organic solvent (Scheme 68). Racemic 2-amino-1-alcohol esters are not easily synthesised, so are unpractical substrates. Attempts to resolve racemic 2-amino-1-alcohols by enzymatic transesterification in ethyl acetate resulted, however, only in non-stereospecific acylation of the more nucleophilic amino group.



(a) R= C_2H_5, R^1= CH_3
(b) R=C_2H_5, R^1=n-C_3H_7
(c) R= CH_3, R^1=CH_3

Scheme 67[148]

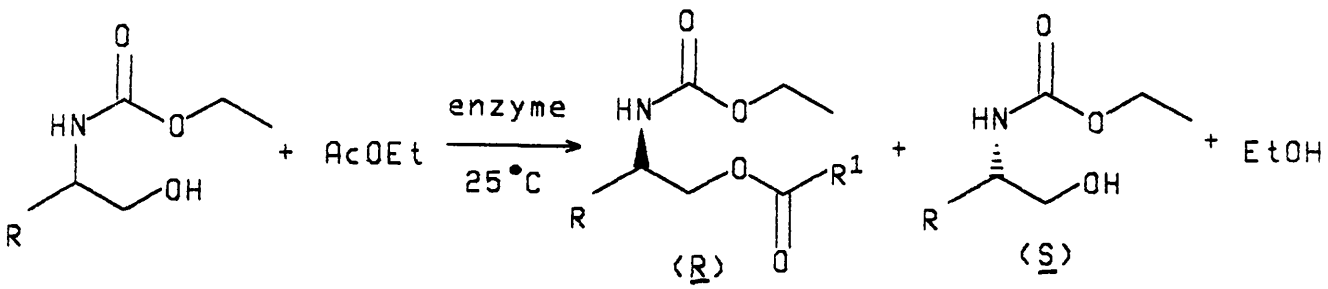


(R,S) (S) (R)

Scheme 68

Although many commercial lipases were tried for the hydrolyses shown in Scheme 68, only three - pancreatin, steapsin, and Lipase Amano P gave satisfactory results.

Lipase catalyzed transesterification in ethyl acetate was used to resolve N-alkoxy-carbonyl derivatives of 2-amino alcohols (Scheme 69).

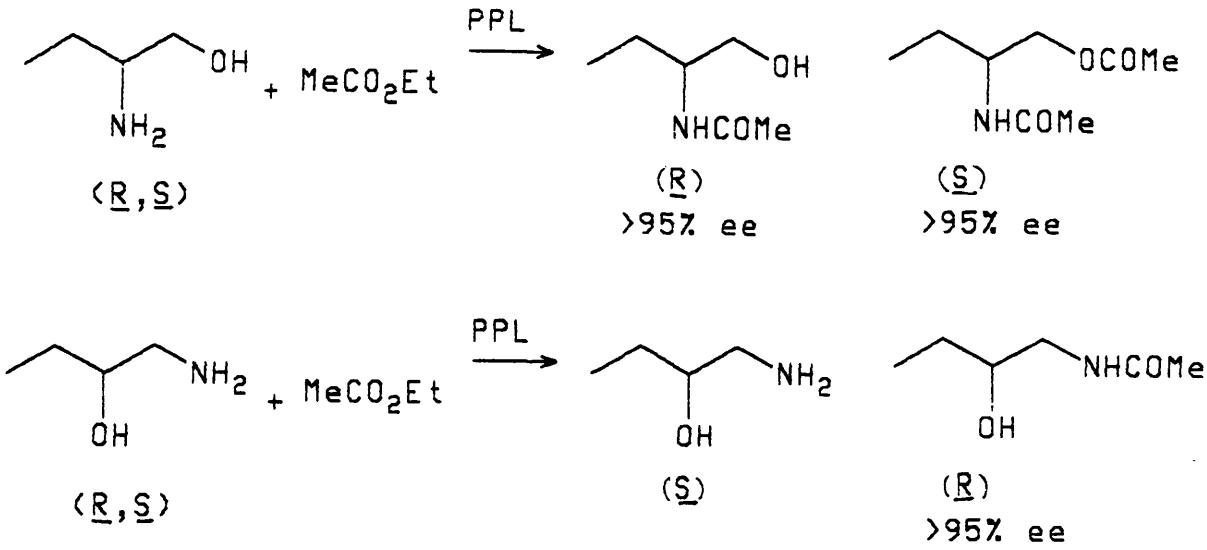


(R,S)

Scheme 69

However, Gotor and co-workers found that PPL in ethyl acetate catalyzed the enantioselective acylation of the amino group of 1-aminopropan-2-ol and the N- and O-acylation of 2-aminobutan-1-ol (Scheme 70).[149]

Klibanov and co-workers found that the chemoselectivity of the enzymatic acylation could be readily controlled by the nature of the acyl moiety.[150] They studied 6-amino-1-hexanol as a model bifunctional compound. Using 2-chloroethyl butyrate as the acylating agent, the OH group was acylated 37 times faster than the NH_2 group. In contrast, when the 2-chloroethyl ester of N-acetyl-L-phenylalanine was the acylating moiety, the reactivity of the amino group was five times that of the hydroxyl group. This observation occurred with several enzymes - Aspergillus niger lipase, PPL and Pseudomonas sp. lipoprotein lipase.



Scheme 70

In parallel with amino-alcohols, diamines have importance in organic synthesis. Optically active diamines would also be useful in our studies of enzyme inhibitors of polyamine biosynthesis. Studies have been carried out to optimise conditions for monoacylation of diamines, and a few chiral diamines have been tested with PPL to discover if stereoselective monoacylation occurs. This work is reported in the following two sections.

7.2 Monoacylation of Diamines

Monoacylations of diamines were first studied using the model compound putrescine (28) since it was readily available from Aldrich and was inexpensive. The acylation reaction was performed at different temperatures, to observe its effect on selectivity. These reactions were all performed using PPL as the catalyst, and ethyl acetate as the acylating agent and solvent. At 15°C, the reaction was slow - after 9 days only monoacylated material was observed with an isolated yield of 41% being achieved. At 25°C, again only monoacylation occurred during the first 48 hours and the isolated yield was 43%. After this period, the amount of monoacylated putrescine decreased and diacylated material became visible. The reaction was a great deal faster at 35°C - even at 16 hours, diacylated putrescine was present. By 25 hours, most of the putrescine had been diacylated. At 35°C, there is little selectivity for monoacylation. The temperature of 25°C was chosen for the further studies with diamines, as it gave the best results with regard to selectivity combined with reasonable rate of reaction.

Different acylating agents were tested with putrescine as the substrate in order to monitor the various rates of reaction and thus perhaps gain an insight into the active site of the enzyme. Five esters were studied - ethyl formate, ethyl acetate, vinyl acetate, ethyl propionate, and ethyl benzoate. The reactions were all run at 25°C and were monitored by t.l.c. It was found that the transacylation reactions of putrescine with ethyl formate and vinyl acetate were both much faster than with ethyl acetate; both mixtures contained primarily diacylated material after five hours. The reaction mixture containing

vinyl acetate was contaminated with polymeric material. In contrast, use of ethyl propionate and ethyl benzoate led to much slower transacylation. After 15 days, the reaction in ethyl propionate contained only starting material and monoacylated material, which was subsequently isolated to yield a 41% conversion into N-propionylputrescine hydrochloride. In an attempt to recrystallise the monopropionyl putrescine hydrochloride, the compound was dissolved, heated in 95% aqueous ethanol, and a few drops of acetone were added. The recovered salt was analysed and found to be putrescine dihydrochloride, suggesting that the monoacylated product was unstable. Ethyl benzoate was a poor acylating agent also. After 16 days, only starting material was present. A small quantity of monoacylated putrescine was visible after 30 days. Thus acylation with smaller agents appears to be faster than with larger ones. As expected, the irreversible nature of the reaction with vinyl acetate ensures that a faster rate is attained. Obviously, for an attractive reaction the polymerisation side-reaction must be eliminated. This could perhaps be achieved by reducing the quantity of vinyl acetate present by dilution with an inert solvent. When this was tried, however, the polymerisation by-products were still observed. Further work is, therefore, required in this area.

After testing different acylating agents, it was decided to investigate the effect of the alkyl chain of the ester on the rate of transacylation. In this experiment the alkyl group was varied while retaining the same acylating group, the acetate moiety. Six solvents were employed, viz. methyl acetate, ethyl acetate, n-propyl acetate,

i-propyl acetate, n- butyl acetate and phenyl acetate. The substrate used was again putrescine, the temperature was 25°C, the enzyme was PPL and the reactions were monitored by t.l.c. Use of methyl acetate gave slightly slower acylation than ethyl acetate, a result which was unexpected and, as yet, unexplained. The larger n-butyl alkyl chain renders the reaction slower than the shorter n-propyl chain, which in turn is faster than the branched i-propyl ester. The phenyl acetate was the slowest acylating agent. The results demonstrate that different rates of acylation can be achieved by altering the alcohol portion of the ester.

The water content of the reaction mixture is believed to be crucial in the 'ester synthesis' mode of the enzyme reaction. It has been suggested that any water present in the solvent inhibits the enzyme catalysing the synthesis of the ester, and activates the hydrolysis mode of action. Samples of ethyl acetate containing various concentrations of water were prepared - 0.1%, 0.2%, 0.4%, 0.8%, and 1.6%, and putrescine was dissolved in these samples. PPL was incubated in the samples at 25°C and the reactions were monitored. It was found that the greater the amount of water present in the ethyl acetate, the greater the proportion of diacetylated material was present in the solution. This fact could be interpreted as evidence of an increased concentration of water causing a corresponding increase in the rate of reaction. On removing the flasks from the incubator, however, it was observed that crystals had formed on the enzyme's surface in vessels containing ethyl acetate with 0.1% and 0.2% water content. No crystals were present in the flask with 1.6% water, while some small crystals were present in the 0.4% and 0.8% flasks.

These crystals were assumed to be diacetylated putrescine, as both putrescine and monoacetylated putrescine are soluble under these conditions. Some crystals were subsequently analysed and were indeed found to be diacetylated putrescine; hence the effect being observed was a solubility factor and not necessarily a rate factor. The results of this experiment remain inconclusive. A greater variation in the water concentration would, perhaps, give clearer results, so this was attempted next. PPL was incubated in flasks containing putrescine as substrate and ethyl acetate as solvent with 0, 6, 12, 25, and 50% water. After 5 days, only diacylated material was present in the flasks containing 25 and 50% water, all three compounds (starting material, mono- and diacylated material) were present in the flasks with 6 and 12% water, and the flask containing no added water consisted mainly of starting material and monoacylated product. These observations suggest that water increased the rate of formation of the diacylated product. However, in no case did the proportion of monoacylated product exceed 45%.

Although in our early experiments monoacylation was obviously taking place, low conversions (between 40 and 45%) were observed. It was suggested that the yields could be improved by increasing the solvent polarity; that is, imitating water without invoking the hydrolysis mode of reaction. To this end, a 50:50 mixture of DMSO and ethyl acetate was tried: the amount of conversion into monoacylated putrescine remained unchanged however.

The reactions described thus far used porcine pancreatic lipase as the enzyme, due to its ease of availability and relative

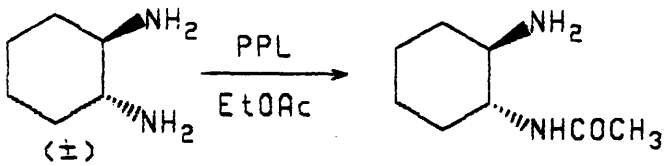
inexpensiveness. As an alternative, lipase from Candida cylindracea and Acylase 1 enzyme (which normally catalyses the hydrolysis of amides) were tested to analyse their capabilities of functioning in anhydrous ethyl acetate to catalyse the acylation of putrescine. Both enzymes gave disappointing results - the monoacylation reaction occurred extremely slowly, and no diacylated product was observed, even after 72 days!

Under the correct conditions, the PPL catalysed reaction could be a useful method for preparing monoacylated polyamines, albeit in moderate yield.

7.3 Attempts to Resolve Racemic Diamines

Attempts were made to resolve racemic mixtures of diamines using the enzymatic monoacylation. It was hoped that the enzyme would show stereoselective properties and hence monoacylate one enantiomer preferentially. The optically active monoacylated product could then be separated from the starting material, which would contain an excess of the other unreacted enantiomer. In order to achieve maximum optical purity, the reaction must proceed to less than 50%. This resolution was attempted on three racemic diamines and one meso diamine with the following results:

(i) (±)-trans-1,2-Diaminocyclohexane

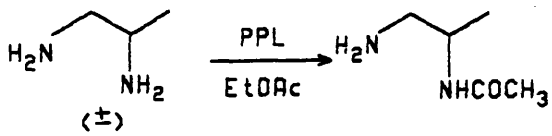


(±) (155) (156)

Scheme 71

By monitoring the reaction of trans-1,2-diaminocyclohexane (155) with PPL in ethyl acetate by t.l.c. (Scheme 71), it was revealed that monoacylation occurred after 2 days and diacylated material was formed after 6 days. The reaction mixture was worked up after 3, 6, and 12 days. In all cases, both the recovered starting material and the monoacylated product had zero optical rotations (cf. optically pure starting material has $[\alpha]_D = -17.18°$). Thus the enzyme appeared to show no enantiomeric selectivity under these conditions.

(ii) (±)-1,2-Diaminopropane

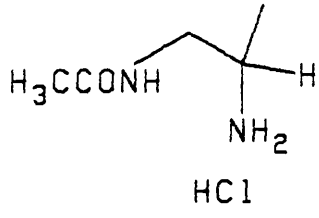


(±) (157)

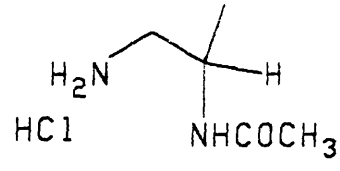
Scheme 72

The reaction of 1,2-diaminopropane (157) with ethyl acetate catalysed by PPL was followed by t.l.c. (Scheme 72). This showed that only monoacylated product was formed. The reaction was terminated after 3, 6 and 22 days and the product and remaining starting material isolated. Again, both compounds displayed zero readings in the polarimeter.

Only one monoacylated product was formed, believed to be (159). Thus regioselective acylation has been achieved. The two possible monoacylated products (158) and (159) cannot be distinguished by m.s., i.r. or micro-analysis. The 200 MHz ^{1}H n.m.r. spectra, however, did indicate the structure of the product which had been formed.



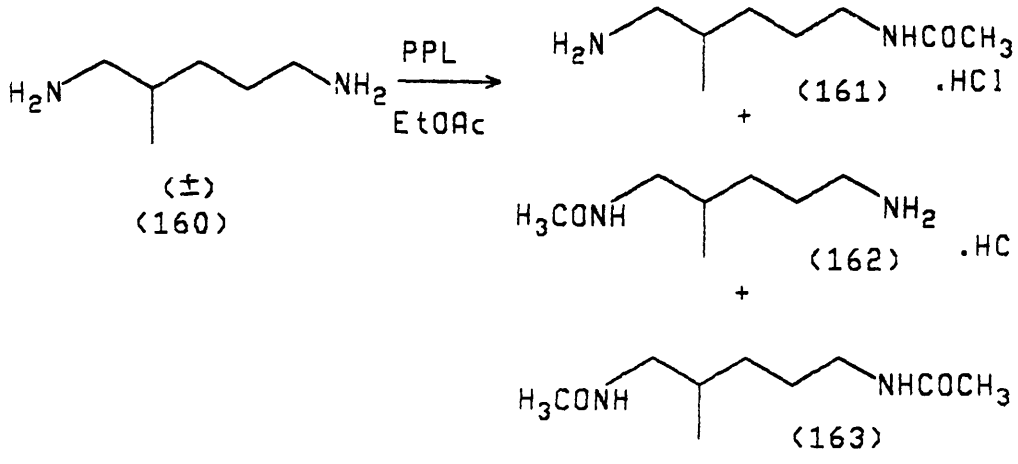
(158)



(159)

In the spectrum of the dihydrochloride salt of recovered starting material, the proton signals appeared at δ 3.60 (for the methine H), 3.16 (for the methylene 2H) and 1.30 p.p.m. (for the methyl group 3H). In the product, the methine and methylene protons appear as a complex signal at ca. δ 3.27 p.p.m., and the methyl protons at δ 1.12 p.p.m. The signals of the methine and methyl protons are most affected then by the presence of the acetyl group. This suggests that the product is (159).

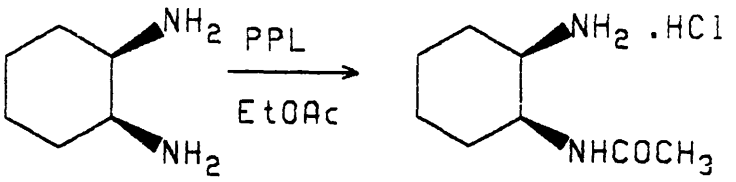
(iii) (±)-2-Methylcadaverine



Scheme 73

In the reaction of (±)-2-methylcadaverine (160) with ethyl acetate catalysed by PPL, diacylated and two different monoacylated products were formed after 7 days (Scheme 73). Since the monoacylated compounds, believed to be (161) and (162), had very similar Rf values, the products of the reaction were separated on 0.25 mm silica preparative t.l.c. plates. No separation could be achieved when thicker silica plates were employed. The plates were developed in EtOAc-i-PrOH-NH_3 in the ratio of 9:7:4, and eluted using methanol with a few drops of ammonia. The products were identified as being two monoacylated and one diacylated compounds. Due to the small quantities of material recovered, no microanalysis data could be attained. From the unclear ^1H n.m.r. spectra, it is not possible to assign structures to the two monoacylated products. Yet again, no optical activity was observed in any of the products.

(iv) meso-cis-1,2-Diaminocyclohexane



(164) (165)

Scheme 74

The meso compound cis-1,2-diaminocyclohexane (164) was incubated with PPL in ethyl acetate, and the reaction was monitored by t.l.c. (Scheme 74). As in the case of (155), monoacylation was first observed after 2 days and diacylation was first seen after 6 days. The reaction mixture was worked up after 6 and 12 days, but again the product had no rotation.

A recent publication by Klibanov and co-workers states that the solvent plays a crucial role in the enzymic resolution of racemic amines.[151] It was found that the enantioselectivity of the enzyme subtilisin was highly influenced by the solvent and varied from nearly unity for toluene, octane and ethyl acetate to 7.7 for 3-methyl-pentan-3-ol, when the substrate was α-methylbenzylamine. (Enantioselectivity was measured as $\frac{V_S}{V_R}$, where V is the initial reaction rate). Therefore, this solvent was integrated into the PPL system. The substrate was dissolved in 3-methylpentan-3-ol containing 2 molar equivalents of ethyl acetate. This method was applied to diamines (155) and (157). trans-1,2-Diaminocyclohexane (155) showed no difference in the results with ethyl acetate - still no optical activity was observed in products or starting materials recovered. Even with this solvent, it appears that the enzyme does not distinguish between the two trans enantiomers of the compound.

In the case of 1,2-diaminopropane (157), a small rotation of -8.7° was observed for the monoacylated product after incubation with PPL in 3-methylpentan-3-ol for 6 days. (The $[\alpha]_D$ of this product is not known). Thus, some enantioselectivity is in operation. No rotation was noted, however, in the recovered starting material. Experiments to determine the ee by addition of camphorsulphonic acid were inconclusive.

Due to lack of time, no further investigations were performed. More studies into the enzymic monoacylation process should, however, be carried out. Some experiments employing different solvents should be performed to find the best one for greatest enantioselectivity. The possibility of using the system to resolve racemic diamines is viable once a suitable solvent is found. It is in this direction that the research must now proceed.

CHAPTER EIGHT

EXPERIMENTAL

8.1 General

All melting points were measured on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with an Optical Activity Ltd. AA 10 Polarimeter. Infra red spectra were obtained on a Perkin Elmer 580 spectrophotometer. Nuclear magnetic resonance spectra were recorded with a Perkin Elmer R32 spectrometer operating at 90 MHz (δ_H), a Varian XL-100 spectrometer operating at 25 MHz (δ_C), a Bruker WP200-SY spectrometer operating at 200 MHz (δ_H), 50 MHz (δ_C) and 30.72 MHz (δ_D). Spectra were recorded for solutions in deuteriochloroform unless otherwise stated, with tetramethylsilane as internal standard. Mass spectra were obtained with A.E.I. MS 12 or 902 spectrometers.

T.l.c. was carried out on Kieselgel G plates of 0.25 mm thickness and developed as stated. The alkaloids were detected by the modified Dragendorff reagent,[152] or by Ehrlich's reagent.[153]

Radiochemicals were purchased from Amersham International. Radioactivity was measured with a Philips PW 4700 Liquid Scintillation Counter using Ecoscint solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each determination. Radioactive samples were counted in duplicate. A Panax thin-layer scanner RTLS-1A was used for radioscanning of t.l.c. plates.

Tetrahydrofuran (THF) was dried by distillation from potassium hydroxide and then from sodium-benzophenone under nitrogen prior to use. Organic solutions were dried with anhydrous sodium sulphate and solvents were evaporated off under reduced pressure below 50°C.

8.2 Experimental to Chapter 3

[2,2,4,4-$^{2}H_{4}$]-1,5-Diaminopentane dihydrochloride (56).- The ^{2}H-labelled cadaverine (56) was prepared according to the method of Robins and Sheldrake.[65] The ^{1}H n.m.r. spectrum indicated that the dihydrochloride contained ca. 92% $^{2}H_{4}$ species; δ_{H} (90 MHz) ($D_{2}O$) 3.42 (s, 4H) and 1.85 p.p.m. (s, 2H).

[3,3-$^{2}H_{2}$]-1,5-Diaminopentane dihydrochloride (57).- Preparation of the title compound was performed by the method of Robins and Sheldrake.[65] The ^{1}H n.m.r. spectrum showed that the salt contained ca. 98% $^{2}H_{2}$ species; δ_{H} (90 MHz) ($D_{2}O$) 4.16 (t, 4H, J 6 Hz) and 2.30 p.p.m. (t, 4H, J 6 Hz).

(R)-[1-^{2}H]-1,5-Diaminopentane dihydrochloride (35).- The title compound was prepared by the method of Spenser and Richards,[48] employing enzymatic decarboxylation of L-lysine in $D_{2}O$ by L-lysine decarboxylase. From ^{1}H n.m.r. spectroscopic data, the deuterium content was estimated to be ca. 90%.

(S)-[1-^{2}H]-1,5-Diaminopentane dihydrochloride (36). The enantiomer was prepared by enzymatically decarboxylating DL-[2-^{2}H]lysine in water using L-lysine decarboxylase.[48] The ^{1}H n.m.r. spectrum was identical to that of (R)-[1-^{2}H]cadaverine dihydrochloride. The deuterium content was estimated to be ca. 85%.

(R)-[2-^{2}H]-1,5-Diaminopentane dihydrochloride (89) and (S)-[2-^{2}H]-1,5-Diaminopentane dihydrochloride (90).- The enantiomerically ^{2}H-labelled cadaverines (89) and (90) were synthesised according to the procedure of Robins and Sheldrake. I should like to express my gratitude to my colleague, Dr. G.N. Sheldrake, who provided samples (89) and (90).

[1,5-$^{13}C_2$; 2,2,4,4-$^2H_4$]-1,5-Diaminopentane dihydrochloride (99).- Propane-1,3-diol (5g, 66 mmol) was dissolved in anhydrous THF (100 ml) and the solution cooled to -78°C, under a dry argon atmosphere. Methanesulphonylchloride (10.15 ml, 132 mmol) was added with stirring, then triethylamine (13.3g, 132 mmol) was added slowly over 5 min. The solution was allowed to reach room temperature overnight. The mixture was then poured into ice/water (200 ml) and extracted with methylene chloride (3 x 100 ml). The combined extracts were dried (Na_2SO_4), filtered and evaporated to dryness to leave a yellow oil. The oil was crystallised using ethyl acetate to give the dimesylate (10.87g, 72%); m.p. 41-43°C; ν_{max} (KBr disc) 3030, 1345, 1173, 980 cm^{-1}; δ_H (90 MHz) 4.39 (t, 4H, J = 7 Hz), 3.06 (s, 6H), 2.21 (quintet, 2H, J = 7 Hz); m/z 202, 175, 111, 97, 79 (100), 57, 29, 15.

(Found: C, 25.80; H, 5.18; S, 27.39. $C_5H_{12}O_6S_2$ requires C, 25.86; H, 5.17; S, 27.59%).

Propane-1.3-diol dimethanesulphonate (1.60g, 7.0 mmol) (101) in dry DMSO (5 ml) was added by syringe to a stirred solution of ^{13}C-labelled sodium cyanide (0.76g, 15.2 mmol) in DMSO (25 ml) at 85-90°C, under a dry argon atmosphere. Stirring was continued at 85-90°C for 3h and then the solution was allowed to cool overnight. The solution was diluted with methylene chloride (100 ml) and washed thoroughly with brine (5 x 50 ml). The organic solution was dried, filtered and evaporated to dryness to leave a yellow oil (0.42g, 63%); b.p. 90°C (0.4 mm Hg); ν_{max} (thin film) 2250 cm^{-1}; δ_H (90 MHz) 1.99 p.p.m. (s, 2H); m/z 96, 54, 41, 28.

[1,5-$^{13}C_2$]Glutaronitrile (0.40g, 4.2 mmol) (102) DBU (1.39g, 9.2 mmol) and D_2O (30 ml) were stirred vigorously in a stoppered flask for 5 days. The solution was neutralised with CH_3CO_2D; extracted with methylene chloride (5 x 10 ml) and the combined extracts washed with brine (5 ml). The organic layer was then dried, filtered and evaporated to dryness (0.174g, 42%). The product showed a $^2H_4$ content of 85%; ν_{max} ($CC\ell_4$) 2950, 2255 and 1430 cm^{-1}; δ_H (90 MHz) 1.99 p.p.m. (s); m/z 100, 56, 53, 44.

Several attempts to improve the deuterium exchange were made on unlabelled glutaronitrile. The chemical yields and exchanges are shown below:-

0.53 equivalents of DBU $\Longrightarrow$ 78% D exchange and 64% chemical yield

1 equivalent of DBU $\Longrightarrow$ 85% D exchange and 50% chemical yield

2 equivalents of DBU $\Longrightarrow$ 90% D exchange and 38% chemical yield

Since material with > 90% deuterium exchanged was desired, 2.2 equivalents of DBU were used.

[1,5-$^{13}C_2$; 2,2,4,4-$^{2}H_4$]Glutaronitrile (130 mg, 1.3 mmol) (103) was reduced by BH_3.THF as described earlier, and the product was recrystallised from aqueous ethanol and acetone (85 mg, 37%); ν_{max} (KBr disc) 3420, 3030, 1600, 1570, 1465 and 1405 cm^{-1}; δ_H (90 MHz) (D_2O) 3.28 (s, 4H), 1.98 (trace), 1.70 p.p.m. (s, 2H); δ_C (25 MHz) (D_2O) 43.5 p.p.m.; m/z 105, 104, 103, 90, 60, 44, 38 and 31.

[1-amino-^{15}N, 1-^{13}C]-1,5-Diaminopentane dihydrochloride (29). - The labelled cadaverine (29) was prepared according to the method of Rana and Robins.[36,96]

3,3-Dimethylcadaverine dihydrochloride (108).- 2,2-Dimethylpropane-1,3-diol (2.71g, 26 mmol) was dissolved in anhydrous THF (50 ml) and the solution was cooled to -78°C. Mesyl chloride (4.0 ml, ρ 1.48, 52 mmol) was added with stirring and then triethylamine (5.25g, 52 mmol) was added slowly. The mixture was allowed to reach room temperature overnight, then was poured into ice/water (100 ml) and extracted with methylene chloride (3 x 75 ml). The combined extracts were dried (Na_2SO_4), filtered and evaporated to dryness to leave a yellow oil, which was crystallised from diethyl ether (5.66g, 92%), m.p. 73-75°C; ν_{max} (KBr disc) 3020, 1350, 1175, 957 and 950 cm^{-1}; δ_H (90 MHz) 4.03 (4H, s), 3.03 (6H, s) and 1.06 p.p.m. (6H, s); m/z 205, 175, 151, 97, 85, 79, 55 (100) and 41. (Found: C, 32.5; H, 6.20; S, 24.71. $C_5H_{16}S_2O_6$ requires C, 32.29; H, 6.20; S, 24.63%).

2,2-Dimethylpropane-1,3-diol dimethanesulphonate (5.03g, 19.3 mmol) in dry DMSO (12 ml) was added by syringe to a stirred solution of NaCN (2.08g, 42.8 mmol) in DMSO (60 ml) at 85-90°C under a dry argon atmosphere. The reaction mixture was stirred at this temperature for one week, then allowed to cool. The solution was diluted with methylene chloride (250 ml) and the mixture washed thoroughly with brine (5 x 100 ml). The organic solution was dried (Na_2SO_4), filtered and evaporated to dryness to afford a yellow oil (1.01g, 43%). b.p. 185-90°C at > 1 mm Hg; ν_{max} (thin film) 2970, 2940, 2241, 1355, 1175 and 1060 cm^{-1}; δ_H (90 MHz) 2.42 (4H, s) and 1.25 p.p.m. (6H, s); m/z 123, 107, 82 (100), 55 and 41.

3,3-Dimethylglutaronitrile (1g, 8.2 mmol) was dissolved in anhydrous THF (15 ml) and brought to reflux under a dry argon atmosphere. Borane THF (1M, 18.5 ml, 18.5 mmol) was added dropwise by syringe. Heating at reflux was continued for 15 min and then the solution was allowed to cool to room temperature. Methanolic hydrogen chloride (1M, 20 ml) was added with vigorous evolution of hydrogen being observed after each addition and after a short induction period. When the addition was complete, the apparatus was rearranged for distillation. The boron residues azeotroped as methyl borate with the THF at 60°C. Final traces of methyl borate were removed by addition of a few ml of methanol and evaporation under reduced pressure. The crude product was then dried in vacuo over P_2O_5 for 24h. The crude solid was recrystallised from absolute ethanol, water and acetone (1.06g, 64%). m.p. 247-249°C. ν_{max} (KBr disc) 3010, 2920, 1610, 1515 and 1410 cm^{-1}; δ_H (90 MHz) (D_2O) 4.93 (HOD), 3.33

(4H, m), 1.89 (4H, m) and 1.24 p.p.m. (6H, s); m/z 167, 129, 112, 69, 55 and 36 (100). (Found: C, 32.68; H, 9.72; N, 16.38. $C_7H_{20}N_2C\ell_2$. $NH_4C\ell$ requires C, 32.75; H, 9.36; N, 16.37%).

[1,5-^{14}C]-3,3-Dimethylcadaverine dihydrochloride. – The title compound was prepared by the same method as unlabelled dimethylcadaverine dihydrochloride, except that $Na^{14}CN$ was used to displace the mesylate group.

Overall radiochemical yield = 19%.

3-Fluorocadaverine dihydrochloride (111).- Diethylaminosulphur trifluoride (DAST) (1.5 ml, 1.83g, 11.4 mmol) was added via syringe to $CH_2C\ell_2$ (20 ml) at -78°C with stirring under a nitrogen atmosphere. Diethyl 3-hydroxyglutaroate (2g, 9.80 mmol) in $CH_2C\ell_2$ (10 ml) was added dropwise over 20 mins. The reaction mixture was allowed to reach room temperature overnight.

The reaction mixture was poured into ice/water (50 ml) and ethyl acetate (50 ml) and then transferred to a large conical flask containing $NaHSO_4$ (5g). After the mixture had been stirred for 1h, the layers were separated. The aqueous layer was washed with ethyl acetate (3 x 30 ml) and the ethyl acetate layer was washed with water (2 x 20 ml). The combined ethyl acetate layers were dried (Na_2SO_4) and evaporated to dryness to yield the product as an oil, which was distilled (1.88g, 93%). b.p. 70°C at 0.2 mm Hg; ν_{max} (thin film) 2980, 1740, 1275, 1155 and 1030 cm^{-1}; δ_H (200 MHz) 5.25 (H, complex), 4.10 (4H, m, J 7.1), 2.72 (4H, m) and 1.19 p.p.m. (6H, m, J=7.1). δ_F (188 MHz) ($CFC\ell_3$) -185.76 p.p.m.; m/z 161, 141, 113, 85, 73 and 29 (100). (Found: C, 52.64; H, 7.24. $C_{19}H_{15}O_4F$ requires C, 52.43; H, 7.28%).

A solution of diethyl 3-fluoroglutaroate (0.41g, 2.0 mmol) in dry toluene (10 ml) was cooled to -30°C (dry ice/acetone) and DIBAL (7 ml, 1.5M) was added with stirring under nitrogen. The mixture was stirred at -30°C for 30 min and then allowed to reach 0°C. Ethyl acetate (1 ml) was added and the mixture was poured onto a suspension of Celite (10g) in acetone. Methanol (10 ml) was added with vigorous stirring; heat was generated and gas was evolved. The mixture was stirred until it gelled. The gel was left for 1h and then water (20 ml) was added to break up the gel. The mixture was filtered, washed with water (2 x 10 ml), then with methanol (3 x 10 ml) and the solvent was removed in vacuo, then the residue was azeotroped with benzene (4 x 2 ml) (0.17g, 70%). b.p. 154°C at 0.5 mm Hg; ν_{max} (thin film) 3360, 2940, 1366 and 1050 cm^{-1}; δ_H (200 MHz) 4.89 (1H, complex), 3.75 (4H, m), 2.82 (2H, b.s.) and 1.67 p.p.m. (4H, m); δ_F (188 MHz) ($CFC\ell_3$) -185.31 p.p.m.; m/z 104 (M^+-H_2O), 103 (M^+-F), 86, 69, 55, 43, 32 and 19 (100). (Found: C, 49.14; H, 9.20. $C_5H_{11}O_2F$ requires C, 49.18; H, 9.02%).

3-Fluoropentane-1,5-diol (0.54g, 4.4 mmol) was dissolved in THF. A solution of hydrazoic acid in benzene (0.79M, 13.4 ml, 10.6 mmol) was added, followed by a solution of di-isopropyl azodicarboxylate (1.99g, 9.7 mmol) in THF (10 ml). Triphenylphosphine (5.09g, 19.36 mmol) in THF (60 ml) was added. The rate of addition was kept so as to maintain the temperature at 40°C. The reaction mixture was stirred at room temperature for 1h, then heated at 50°C for 3h. Water (2 ml) was added, and the solution was stirred at 50°C for a further 3h.

The solvents were removed in vacuo and the residue was partitioned between 1M HCℓ (80 ml) and $CH_2C\ell_2$ (80 ml). The aqueous layer was further extracted with $CH_2C\ell_2$ (2 x 80 ml). The aqueous layer was evaporated in vacuo leaving 3-fluorocadaverine dihydrochloride which was recrystallised from aqueous ethanol/acetone (1:1) (270 mg, 32%). m.p. 258-260°C. ν_{max} (KBr disc) 3430, 3000, 1475, 1150 and 845 cm^{-1}; δ_H (200 MHz) (D_2O) 4.80 (1H complex), 4.67 (HOD), 3.07 (4H, t, J 7.2 Hz) and 2.02 p.p.m. (4H, m); δ_F (188 MHz) ($CFC\ell_3$) -184.52 p.p.m.; m/z 102, 81, 56, 42, 35 and 30 (100). (Found: C, 30.92; H, 7.61; N, 14.66. $C_5H_{15}FN_2C\ell_2$ requires C, 31.08; H, 7.77; N, 14.51%).

Feeding Methods and Extraction and Separation of the Alkaloids

Feeding Methods.-

The plants were grown in pots in standard compost in a greenhouse. Eight plants were used for most of the experiments. To each precursor was added a sample of ^{14}C- or ^{3}H-labelled cadaverine dihydrochloride (1 or 2 μCi), and the compounds were divided into equal portions. Each portion was dissolved in sterile water and fed to the plants by either the wick (S. microphylla and A. foetida) or the xylem-pricking (L. luteus) method, on alternate days for a period of 10-14 days. After a further period of 10-14 days, the plants were harvested, and the alkaloids were isolated by a standard method, described below.

Fresh leaves, stems and roots (140g) of the plants were finely chopped and blended with methanol. The blended extracts were filtered and the methanolic filtrates were concentrated under reduced pressure. The resulting green residue was dissolved in dichloromethane (100 ml) and extracted with 1.5M sulphuric acid (3 x 100 ml). The combined aqueous extracts were stirred at room temperature for 1½h with zinc dust (5g) to reduce any N-oxides formed. The aqueous mixture was filtered through a pad of Celite 535 which was washed with further acid (100 ml) and water (2 x 100 ml). The combined aqueous solutions were cooled in ice/water and basified with concentrated ammonia solution. The resultant alkaline solution was extracted with dichloromethane (4 x 300 ml), and the combined extracts were dried, filtered and evaporated to dryness to leave a viscous oil (200 mg).

Incorporation figures for each experiment are provided in the relative sections of Chapter Three.

The total alkaloidal mixtures were separated using Kiesgel G silica plates of 0.25 mm thickness. The plates were developed in chloroform/methanol/ammonia (85:14:1) and visualized with u.v. or Dragendorff's reagent. The bands were scraped off and eluted with methanol containing a few drops of ammonia. The solutions were then filtered, dried and refiltered, and evaporated to dryness to yield the isolated alkaloid. Sophora microphylla produced matrine, anagyrine, N-methylcytisine and cytisine; Anagyris foetida yielded anagyrine and N-methylcytisine while Lupinus luteus gave sparteine and lupinine.

Matrine: R_F 0.88, $[\alpha]_D$ = 39° (lit. value[154] 40.9° in water), (Found $\underline{M}^+$, 248.1882; $C_{15}H_{24}N_2O$ requires $\underline{M}$, 248.1888); Anagyrine: R_F 0.80, $[\alpha]_D$ = -164° (lit. value[154] -165. ° in ethanol), (Found $\underline{M}^+$, 244.1565; $C_{15}H_{20}N_2O$ requires $\underline{M}$, 244.1576); N-Methylcytisine: R_F 0.75, (Found $\underline{M}^+$, 204.1258, $C_{12}H_{16}N_2O$ requires $\underline{M}$, 204.1263); Cytisine: R_F 0.53, (Found $\underline{M}^+$, 190.1098, $C_{11}H_{14}N_2O$ requires 190.1106); Lupinine: R_F 0.52; Sparteine: R_F 0.26.

8.3 Experimental to Chapter 4

Ammodendrine Biosynthesis Investigations

Samples of (R)- and (S)-[1-^{2}H]cadaverine dihydrochloride were prepared as previously described. The deuterium content of the precursors was estimated to ca. 90% and ca. 85%, respectively. Four biosynthetic experiments were performed:

(i) Complete Leaf and Petioles

In each experiment, two "leaf and petioles" from L. polyphyllus were used. The stem was trimmed, and left to stand in a solution of the labelled cadaverine (20 mg) in water (pH = 5.7) for 2 days. When the water level became low, it was topped up with fresh tap water.

After 2 days, the leaf and petioles were cut into small pieces, and ground in 0.5M HCl (15 ml). The mixture was left at room temperature for 30 min, then the solution was made alkaline using 4M NaOH. The mixture was applied to an Extrelute column, eluted with methylene chloride, and the resulting solution was evaporated to dryness. The product alkaloids were transferred to a small vial and

submitted for GLC and GC-MS analysis.

(ii) Leaf discs

Leaflets (2g) from L. polyphyllus plants were cut into 2 mm width pieces. The discs were immersed in tap water (10 ml) in which the labelled cadaverine (10 mg) had been dissolved. Occas ionally, the flask was swirled, and left overnight. The leaf discs were separated from the medium using cheesecloth. The leaf discs were ground with 0.5M HCℓ (15 ml), left for 30 min and made alkaline using 4M NaOH. The mixture was applied to an Extrelute column, eluted with $CH_2C\ell_2$ and evaporated to dryness. The separated medium was made alkaline and applied to a column in the same way. The combined alkaloidal extracts were transferred to a vial and analysed by GLC and GC-MS.

(iii) Cell-free Reaction with Diamine Oxidase Enzyme

Samples of labelled cadaverine were tested with two DAO enzymes - from Lupinus arboreus and from Pisum sativum. In each experiment, 200 μℓ of DAO enzyme was incubated with cadaverine (5 mg) in buffer (2 ml). The buffer consisted of 0.1M borate buffer (pH 8.5) with a 10 mM pyruvate. The samples were incubated at 37°C in Eppendorf tubes with tops open to the atmosphere. After 18h the solutions were made alkaline using 4M NaOH, and the alkaloids were extracted as described earlier. Again, the alkaloids were transferred to a vial awaiting GLC and GC-MS analysis.

(iv) Enzyme Extraction from L. polyphyllus

Leaf discs (30g) were homogenised in 0.2M pyrophosphate buffer (pH 8.5) (25 ml) by grinding with a small amount of quartz sand in a pestle and mortar. The mortar was kept in ice. The homogenised leaf discs were centrifuged for 5 min at 10,000 rev/min. The supernatant was decanted, and the pH was adjusted to 8.5 using 1M NaOH. Amberlite XAD 2/4 Mesh 20-50 (Sigma) was added to remove the alkaloids, then filtered off using cheesecloth. The resultant enzyme extract (9 ml) was incubated with the labelled cadaverine (10 mg) and pyruvate (10 mg). A control experiment was also set up, where the flask contained only the enzyme. The flasks were incubated overnight at room temperature.

The alkaloids were extracted as in (iii), and analysed by GLC and GC-MS.

8.4 Experimental to Chapter 5

Samples of (R)- and (S)-[1-^{2}H]cadaverine dihydrochloride were prepared as previously described. The deuterium contents of the precursors were estimated to be ca. 90% and ca. 85%, respectively.

The Nicotiana cultures were grown at the A.F.R.C. Food Research Institute at Norwich. The cultures were kept in Gamborg's B50 medium (full strength) (50 ml) and 2% sucrose was added. Every two weeks the cultures were divided.

Extraction of the Alkaloids from Nicotiana Cultures

(Performed at A.F.R.C. Food Research Institute by their research workers).

The roots of the culture were blended in 0.2% H_2SO_4 solution, left for 2h, then the roots were filtered off. After washing with ether, the acidic layer was basified with 1M NaOH to pH6 (± 1). The alkaloids were extracted using ether, dried and evaporated to dryness to leave the alkaloids.

The culture medium was neutralised to pH 6-7 using NaOH. The alkaloidal material was extracted with $CHC\ell_3$ as before.

Separation of Anabasine (by workers at A.F.R.C. at Norwich)

The residue was applied to an Analtech silica taper plate and developed in chloroform/methanol (100/20). The plate was examined under short-wave u.v. and the anabasine zone (R_f ca. 0.34; ochre/pink colour with Konig's reagent) was scraped off. The scrapings were eluted with methanol and after filtration and evaporation of the filtrate to dryness, the residue was redissolved in methanol.

The sample was rechromatographed on an Analtech plate, developing with toluene/acetone/methanol/25% ammonia (4:4.5:1:0.5). The anabasine zone was again scraped off and eluted with methanol, filtered and dried with $CaC\ell_2$ as desiccant. Evaporation yielded anabasine.

8.5 Experimental to Chapter 6

8.5.1 Synthesis of Cadaverine Derivatives with Methyl Substituents

General Procedure:-

The substituted glutaric acid or anhydride (1 mmol) dissolved in THF (20 ml) was brought to reflux under a dry argon atmosphere. Borane THF (1M, 2 mmol) was added dropwise *via* syringe. Reflux was continued for ~ 18h. The reaction mixture was allowed to cool, then water (20 ml) was added *via* syringe. The aqueous layer was then saturated with K_2CO_3 (*ca.* 4g). Diethyl ether (10 ml) was then added to the reaction mixture. The reaction mixture was extracted using diethyl ether (4 x 20 ml); the organic layer was dried (Na_2SO_4) and evaporated to dryness to leave the product as a yellow oil, which was then distilled.

A solution of hydrazoic acid in benzene (1.0M, 2.4 mmol) was added to the substituted diol (1 mmol) in 20 ml THF. A solution of di-isopropyl azodicarboxylate (2.2 mmol) in THF (10 ml) was then added with stirring.

To this mixture was added triphenylphosphine (4.4 mmol) in THF (60 ml). The reaction temperature depends on the rate of addition, which was kept to maintain the temperature at 40°C. The reaction mixture was stirred for 1h at room temperature, then was heated at 50°C for 3h. Water (2 ml) was added, and the solution was stirred at 50°C for a further 3h.

The solvents were removed *in vacuo* and the residue was partitioned between 1M HCℓ (80 ml) and $CH_2C\ell_2$ (80 ml). The aqueous

layer was further extracted with $CH_2C\ell_2$ (2 x 80 ml). The aqueous layer was evaporated in vacuo leaving the dihydrochloride which was recrystallised from aqueous ethanol/acetone (1:1).

Preparation of Hydrazoic Acid:-

A paste was prepared from equal weights of water and sodium azide (32.5g) in a 500 ml three-necked flask. To this paste was added benzene (200 ml) and the mixture was cooled to ~ 7°C. Conc. sulphuric acid (13.3 ml, 0.25 mol) was added slowly to the flask with continued stirring and cooling, keeping the temperature below 10°C. The mixture was stirred for 1h after the acid had been added, then was cooled to 0°C. The organic layer was decanted and dried over Na_2SO_4. The strength of the solution was determined by titration against standard NaOH solution.

3-Methyl-1,5-diaminopentane dihydrochloride (130).-

Using 3-methylglutaric acid, the 3-methylpentane-1,5-diol was obtained as a yellow oil (75% yield), b.p. 160°C at > 1 mm Hg; ν_{max} (thin film) 3360, 2960, 2930, 1455, 1380, 1070 and 1060 cm^{-1}; δ_H (90 MHz) 3.67 (4H, t, J 6 Hz), 3.23 (2H, b.s.), 1.62-1.32 (5H, m) and 0.90 p.p.m. (3H, d, J 6 Hz); m/z 116 (M^+-2H), 100 (M^+-H_2O), 88, 82, 70, 67, 55, 41(100), 39, 31 and 29.

The final product (130) was obtained as a white crystalline solid (24% yield), m.p. 267-268°C; R_f 0.29 (isopropanol/ammonia 5:3); ν_{max} (KBr disc) 3420, 3000, 1595, 1475 and 1400 cm^{-1}; δ_H (90 MHz) (D_2O) 4.93 (HOD), 3.23 (4H, t, J 7 Hz), 1.80 (5H, br s.), and 1.12

p.p.m. (3H, d, J 5Hz); m/z 117, 99, 70, 56, 45, 38, 36, 30(100), 28. (Found: C, 38.10; H, 9.55; N, 14.80; Cℓ, 37.69; $C_6H_{18}N_2Cℓ_2$ requires C, 38.10; H, 9.52; N, 14.81; Cℓ, 37.57%).

2,2-Dimethyl-1-5-diaminopentane dihydrochloride (131).-

Treatment of 2,2-dimethylglutaric acid as described in the general procedure afforded 2,2-dimethylpentane-1,5-diol as a yellow oil (88% yield); b.p. 180°C at > 1 mm Hg; ν_{max} ($CCℓ_4$) 3350, 2950, 2865, 1470, 1360 and 1050 cm^{-1}; δ_H (90 MHz) 3.45 (4H, d, J 6 Hz), 3.30 (2H, b.s.), 1.69 (4H, m), 0.94 (3H, s) and 0.87 p.p.m. (3H, s); m/z 114, 97, 83, 69, 55, 41(100), 29 and 18.

The title compound was prepared (4% yield) as described in the general procedure, m.p. 158-160°C; R_f 0.47 (isopropanol/ammonia 5:3); ν_{max} (KBr disc) 3440, 3020, 2960, 1595, 1500, 1400 and 1375 cm^{-1}; δ_H (90 MHz) (D_2O) 4.80 (HOD), 3.14 (2H, t, J 8 Hz), 3.00 (2H, s), 2.00-1.30 (4H, m), 1.10 (6H, s); m/z 115, 101, 83, 70, 55, 45, 30(100). (Found: C, 40.31; H, 9.79; N, 13.53; Cℓ, 36.40. $C_7H_{20}N_2Cℓ_2$ requires C, 41.38; H, 9.85; N, 13.79; Cℓ, 34.98%).

2,4-Dimethyl-1,5-diaminopentane dihydrochloride (132).-

When 2,4-dimethylglutaric anhydride was treated using the standard procedure, 2,4-dimethylpentane-1,5-diol was produced in 75% yield; b.p. 165°C at > 1 mm Hg; ν_{max} ($CCℓ_4$) 3350, 2955, 2920, 1410, 1360, 1340 and 1075 cm^{-1}; δ_H (90 MHz) 3.47 (4H, d, J 8 Hz), 2.55 (2H, b.s), 1.68 (4H, m), 0.90 (6H, d, J 4 Hz); m/z 114 (M^+-H_2O) 102, 84, 69, 55(100), 41, 31, 18.

Using 2,4-dimethylpentane-1,5-diol, the title compound was obtained as a white crystalline solid (22% yield); m.p. 223-224°C; R_f 0.64 (isopropanol/ammonia 5:3); ν_{max} (KBr disc) 3400, 3020, 2960, 1600, 1565, 1495, 1470, 1180, 1120 and 1010 cm^{-1}; δ_H (90 MHz) (D_2O) 4.83 (HOD), 3.28-2.78 (4H, m), 2.20-1.90 (2H, m), 1.55-1.33 (2H, m), 1.15 (6H, d, J 7 Hz); m/z 131, 113, 101, 98, 70, 59, 42, 36, 30(100). (Found: C, 41.27; H, 9.83; N, 13.80; Cℓ, 34.79. $C_7H_{20}N_2C\ell_2$ requires C, 41.38; H, 9.85; N, 13.79; Cℓ, 34.98%).

3,3-Tetramethylene-1,5-diaminopentane dihydrochloride (133).-

3,3-Tetramethylene glutaric anhydride was subjected to the standard procedure to yield 3,3-tetramethylenepentane-1,5-diol (92% yield); b.p. 160°C at > 1 mm Hg; ν_{max} ($CC\ell_4$) 3330, 2940, 2860, 1445, 1155, 1035 and 1005 cm^{-1}; δ_H (90 MHz) 3.70 (4H, t, J 7 Hz), 2.31 (2H, b.s), 1.53 (6H, m); m/z 140 (M^+-18), 122, 95(100), 81, 79, 67, 55, 41, 31 and 18.

The title compound was prepared from 3,3-tetramethylene-pentane-1,5-diol in 18% yield as a thick oil. R_f 0.32 (isopropanol/ammonia 5:3); ν_{max} (KBr disc) 3400, 2980, 2920, 1470, 1450, 1115 and 870 cm^{-1}; δ_H (90 MHz) (D_2O) 4.93 (DOH), 3.15 (4H, m), 1.75 (4H, m), 1.10 (8H, m); m/z 110, 99, 84, 70, 56, 43, 38, 36 and 30 (100). Satisfactory analytical data could not be obtained.

3,3-Pentamethylene-1,5-diaminopentane dihydrochloride (134).-

3,3-Pentamethylenepentane-1,5-diol was prepared from 1,1-pentamethylene diacetic acid using the general procedure, in 80% yield. b.p. 160°C at > 1 mm Hg; ν_{max} ($CC\ell_4$) 3300, 2920, 2860, 1445, 1320, 1060 and 1025 cm^{-1}; δ_H (90 MHz) 3.80 (4H, t, J 7 Hz), 2.62 (2H, b.s.), 1.70 (2H, t, J 7 Hz), 1.44 (10H, m) and 1.00 p.p.m. (2H, t, J 7 Hz); m/z 154 (M^+-H_2O), 126 (M^+-2H_2O), 108, 93, 81, 67, 56, 40(100), 31 and 18.

3,3-Pentamethylenepentane-1,5-diol was converted in 10% yield to the title compound, as a gum; R_f 0.36 (isopropanol/ammonia 5:3); ν_{max} (KBr disc) 3120, 2930, 1375, 1265 and 1120 cm^{-1}; δ_H (90 MHz) (D_2O) 5.08 (HOD), 3.24 (4H, m), 1.62 (10H, m) and 1.12 (4H, m); m/z 154, 110, 81, 56, 36 and 30(100). Satisfactory analytical data could not be obtained.

3-Hydroxy-3-methyl-1,5-diaminopentane dihydrochloride (137).-

To a stirred solution of dicrotalic acid (5g, 31 mmol) in methanol (250 ml) at 0°C was added dropwise $SOC\ell_2$ (8.81g, 74 mmol). The solution was then evaporated in vacuo to leave a yellow oil, which was distilled in vacuo (5.32g, 91%). b.p. 70°C at 0.5 mm Hg; ν_{max} ($CC\ell_4$) 3510, 2970, 2940, 1730, 1430, 1195 and 1170 cm^{-1}; δ_H (90 MHz) 4.70 (1H, b.s.), 3.70 (6H, s), 2.69 (4H, s) and 1.36 (s, 3H); m/z 175, 117, 85 and 43(100).

3-Hydroxy-3-methylpentane-1,5-diol was prepared from dimethyl dicrotaloate using the standard method. (88% yield); b.p. 165°C at 0.6 mm Hg; ν_{max} ($CC\ell_4$) 3420, 2950, 2920 and

1015 cm^{-1}; δ_H (90 MHz) 3.68 (4H, t, J 7 Hz), 1.68 (2H, b.s.), 1.50 (4H, m) and 0.95 p.p.m. (3H, s); M/z 131 (M^+-3H) 89, 71, 60, 55 and 43(100).

The title compound was synthesised from 3-hydroxy-3-methylpentane-1,5-diol by the general procedure (4% yield); m.p. 249°C (dec); R_f 0.15 (isopropanol/ammonia 5:3); ν_{max} (KBr disc) 3420, 2980, 2920, 1450, 1280 and 1110 cm^{-1}; δ_H (90 MHz) (D_2O) 5.06 (HOD), 3.20 (4H, m), 1.90 (4H, m) and 1.15 p.p.m. (3H, s); m/z 116, 76, 59, 43(100), 36 and 32. (Found: C, 28.28; H, 7.67; N, 15.47. $C_6H_{18}N_2OC\ell_2$ requires C, 35.12; H, 8.78; N, 13.66%).

3-Hydroxy-1,5-diaminopentane dihydrochloride (135).-

Diethyl 3-hydroxyglutarate (0.80g, 4.4 mmol) in anhydrous ether (20 ml) was added to $LiA\ell H_4$ (0.55g, 14.5 mmol) in anhydrous ether (30 ml). The reaction mixture was stirred at room temperature overnight, and then heated at reflux for 3h. After cooling to room temperature, 30% NaOH solution (5 ml) was added dropwise until the inorganic residues turned white and granular. The ethereal solution was carefully decanted from the solid and the residue was extracted with ether (3 x 30 ml); each extract being filtered under vacuum. The combined organic solutions were evaporated to dryness to yield a thick yellow oil (0.33g, 70%). b.p. 170°C at > 1 mm Hg; ν_{max} ($CC\ell_4$) 3360, 2960, 1160 and 1070 cm^{-1}; δ_H (90 MHz) 3.80 (4H, m), 103, 89, 71, 59(100), 42, 38 and 29.

The final product was synthesised from pentane-1,3,5-triol by the standard method in 33% yield. m.p. 225-227°C; R_f 0.21

(isopropanol/ammonia 5:3); ν_{max} (KBr disc) 3400, 3000, 1600, 1400, and 1115 cm^{-1}; δ_H (90 MHz) (D_2O) 4.90 (HOD), 3.23 (4H, t, J 7Hz), 1.69 (1H, m) and 1.10 p.p.m. (4H, m); m/z 120, 82, 71, 56, 44, 38, 36, 30(100) and 18. Satisfactory analytical data could not be obtained.

Attempted synthesis of 3-methoxy-1,5-diaminopentane dihydrochloride (136).-

Silver oxide (4.9g, 2 equiv) was added to diethyl 3-hydroxyglutarate (2g, 9.8 mmol) in methyl iodide (30 ml) and the mixture was heated at reflux for 18h. Excess methyl iodide was removed, the mixture was filtered and the filtrate was dried (anh. $MgSO_4$), filtered and evaporated to yield a yellow oil. The methylation was repeated to yield a greater percentage of methylated product (1.64g). R_f 0.76 (product), 0.65 (starting material) ($CHC\ell_3$). The product was separated from starting material by column chromatography to yield 0.38g (18% yield) of methylated product; ν_{max} (thin film) 2980, 1740, 1445, 1395, 1200, 1095 and 1030 cm^{-1}; δ_H (90 MHz) 4.17 (4H, q, J = 7 Hz), 3.39 (3H, s), 2.58 (4H, d, J 6 Hz) and 1.26 p.p.m. (6H, t, J 7 Hz); m/z 203, 141, 131, 117, 113, 89, 85, 71, 61, 43, 31, 28 and 15.

A solution of diethyl 3-methoxyglutarate (0.22g, 1.1 mmol) in dry toluene (20 ml) was cooled to -30°C and DIBAL (3.5 ml, 5.3 mmol) was added with stirring under N_2. The mixture was stirred at -30°C for 30 min, then the temperature was allowed to reach 0°C. Ethyl acetate (3 ml) was added and the mixture was poured onto a suspension of Celite in acetone. Methanol (10 ml) was added with

vigorous stirring, and the stirring was continued until the mixture gelled. The gel was left for 1h and then water (30 ml) was added to break up the gel. The mixture was filtered, washed with water (2 x 10 ml), methanol (3 x 10 ml) and the solvent was removed in vacuo, then the residue was azeotroped with benzene (4 x 2 ml) to yield a brown viscous oil (0.08g, 59%); ν_{max} (thin film) 3400, 2920, 1460 and 1070 cm^{-1}; δ_H (60 MHz) 3.90 (t), 3.55 (s), 2.47 (b.s.), 1.97 p.p.m. (m). Large impurity at 1.48 p.p.m. No integration data. m/z 134 (M^+), 132, 116, 97, 89, 83, 71, 59, 55, 43 and 41.

Attempted synthesis of 3,3-difluoro-1,5-diaminopentane dihydrochloride (141).-

Thionyl chloride (9.78g, 82 mmol) was added dropwise to a stirred solution of β-ketoglutaric acid (5g, 34 mmol) in methanol (250 ml) at 0°C. The solution was stirred overnight, then evaporated to dryness under reduced pressure to yield a yellow oil which was distilled in vacuo (5.56g, 93%). b.p. 64°C at ~ 5 mm Hg; ν_{max} ($CC\ell_4$) 2970, 1740, 1715, 1660, 1630, 1440, 1320, 1230 and 1140 cm^{-1}; δ_H (90 MHz) 5.15 (small peak), 3.75 (6H, s), 3.67 (4H, s) and 3.26 p.p.m. (small peak); m/z 174 (M^+), 143 ($M-OCH_3$), 101(100), 69, 59, 43 and 31. (Found: C, 48.22; H, 5.70. $C_7H_{10}O_5$ requires C, 48.27; H, 5.79%).

Diethylaminosulphur trifluoride (DAST) (0.38 ml, 2.87 mmol) was added to pentane (20 ml) at 80°C under nitrogen. The apparatus had previously been flame-dried, and flushed with nitrogen. Dimethyl β-ketoglutarate (0.5g, 2.87 mmol) in pentane (5 ml) was added via

syringe dropwise and the solution was stirred overnight.

The reaction mixture was poured into water (50 ml) and ethyl acetate (50 ml) was added. The organic layer was separated and the aqueous layer was extracted with ethyl acetate (2 x 20 ml). The organic layer was then washed with water (4 x 20 ml), dried (Na_2SO_4), and evaporated in vacuo to yield a dark brown oil (0.40g). This brown oil contained starting material and polymeric material but no desired fluorinated product. The reaction was repeated using the following conditions:

(a) temperature - 80°C / time - 28h / solvent - none

(b) temperature - 60°C / time - 2h / solvent - none
followed by
temperature-room temperature/time-18h/solvent-none

(c) temperature-room temperature/time-9 days/solvent-none

Starting material and polymer material only were produced under these conditions.

Attempted synthesis of 2,2-difluoro-1,5-diaminopentane dihydrochloride (142).-

α-Ketoglutaric acid (25.69g, 0.18 mol) was dissolved in a rapidly stirred solution of ethanol. Toluene (80 ml), conc. HCℓ (1 ml) and a further amount of ethanol (40 ml) were added to the reaction mixture, which was heated at 40°C overnight. The azeotropic mixture of ethanol, toluene and water was distilled off under reduced pressure. Toluene (80 ml) and ethanol (125 ml) were added to the residue and the solution was heated at 40°C for 20h. The solvent was removed in vacuo

as before, and the residue was distilled (32g, 90%); b.p. 102°C at 0.35 mm Hg; ν_{max} (thin film) 2985, 1730, 1260, 1205 and 1070 cm^{-1}; δ_H (90 MHz) 4.34 (2H, q, J 8 Hz), 4.18 (2H, q, J 8 Hz), 3.17 (2H, t, J 7 Hz), 2.66 (2H, t, J 7 Hz), 1.38 (3H, t, J 8 Hz) and 1.27 (3H, t, J 8 Hz); m/z 203, 129, 101(100), 55, 29, 27.

The procedure used in the attempted fluorination of dimethyl β-ketoglutarate was also used in the fluorination of diethyl α-glutarate, with the following sets of conditions:

(i) temperature - 80°C / time - 18h / solvent - none;

(ii) temperature - 50°C / time - 18h / solvent - none;

(iii) temperature-room temperature/time-5days/solvent-none;

(iv) temperature - 80°C / time - 1h / solvent - none.

In cases (i), (ii) and (iv) there was evidence for the formation of a small amount of fluorinated product.

R_f (ethyl acetate) 0.66 (product), 0.58 (starting material); ν_{max} (thin film) 2980, 1730, 1640, 1535, 1255, 1095 and 1020 cm^{-1}; δ_H (90 MHz) 4.37 (m), 3.47 (small m), 3.15 (t), 2.65 (t) and 1.36 p.p.m. (m); m/z 226, 203, 129, 101, 73, 55, 43, 29(100) and 18.

Attempted synthesis of 2,2,3,3,4,4-hexafluoro-1,5-diamino-pentane dihydrochloride (145).-

(i) Model Reduction.-

A $LiA\ell H_4$ reduction was performed on glutaric anhydride employing the procedure described in the reduction of diethyl 3-hydroxyglutaroate, earlier in this section to give pentane-1,5-diol

(55% yield); ν_{max} ($CC\ell_4$) 3360, 2940, 2860, 1074 and 1045 cm^{-1}; δ_H (90 MHz) 3.66 (4H, t, J 6 Hz), 2.28 (2H, b.s.) and 1.52 p.p.m. (6H, m); m/z 102, 86, 74, 68, 56, 44, 41 and 31(100).

(ii) Attempted Reduction of 3,3,4,4,5,5-hexafluoroglutaric anhydride (144).-

A standard diborane reduction was performed on compound (144), but did not yield the desired diol. $LiA\ell H_4$ reductions performed using standard and cold (-20°C) conditions did not afford the desired product.

8.6 Experimental to Chapter 7

Standard Procedure for Extraction of Products from Enzyme Reactions

The organic solvent was decanted from the reaction vessel. A mixture of chloroform and methanol (9:1, 20 ml) was added to the enzyme, and the reaction mixture was stirred at room temperature for 1h. The solution was then filtered through Celite, and the combined extracts were dried (Na_2SO_4), filtered, and evaporated to dryness to yield an oil. The oily residue was dissolved in a mixture of hot water and 6M $HC\ell$ (10 ml). This solution was pumped to dryness to give the unreacted diamine dihydrochloride and the monoacylated diamine hydrochloride (and sometimes the diacylated diamine). Extraction of the mixture with propan-2-ol dissolved the acylated compound which was separated by filtration. The volume of the filtrate was reduced and the solution was kept at 0°C overnight. The precipitate was filtered and dried to afford the monoacylated diamine.

Standard Procedure for Enzyme Reaction.-

The diamine (1g) was dissolved in ethyl acetate (10 ml) and porcine pancreatic lipase (PPL) (1g, Activity using triacetin at pH 7.4, 60 min : 13.3 units/mg solid) was added. The flask was shaken in a temperature-controlled water-bath. At intervals, portions were taken out of the reaction mixture and a t.l.c. plate run of the sample in ethyl acetate/isopropanol/conc. ammonia (9:7:4). The progress of the reaction was monitored in this way. At the end of the reaction period, the products were extracted as detailed in the standard procedure.

Temperature Study of the Monoacylation of Putrescine (28).-

(a) 15°C

After a period of 9 days, 41% of the putrescine had been converted to monoacylated putrescine. No diacylated putrescine was observed at this temperature.

(b) 25°C

During the first 48h, only monoacylated putrescine was formed (43% yield). After 48h, diacylated material was observed and the amount of monoacylated putrescine had decreased.

(c) 35°C

Diacylated putrescine was observed after 16h of reaction.

Putrescine dihydrochloride.-

R_f (free base) 0; m.p. > 322°C (dec); ν_{max} (KBr disc) 3080, 1470, 1450 and 1120 cm^{-1}; δ_H (90 MHz) (D_2O) 4.73 (HOD), 3.09 (4H, b.s.) and 1.78 p.p.m. (4H, b.s.); m/z 88, 70, 59, 43 and 30(100). (Found: C, 29.80; H, 8.66; N, 17.35; Cℓ 43.93. $C_4H_{14}N_2C\ell_2$ requires C, 29.81; H, 8.69; N, 17.39; Cℓ, 44.10%).

Monoacetylated putrescine hydrochloride.-

R_f (free base) 0.17; m.p. 136-138°C; ν_{max} (KBr disc) 3460, 3260, 3080, 1650 and 1555 cm^{-1}; δ_H (90 MHz) (D_2O) 4.87 (HOD), 3.33 (4H, m), 2.11 (3H, s) and 1.77 p.p.m. (4H, m); m/z 130, 101, 86, 73, 70, 43, 36 and 30(100). (Found: C, 42.16; H, 8.83; N, 16.05. $C_6H_{15}N_2OC\ell$ requires C, 43.24; H, 9.00; N, 16.81%).

Diacetylated putrescine.-

R_f 0.53; m.p. 133-138°C; ν_{max} (KBr disc) 3300, 1630 and 1540 cm^{-1}; δ_H (200 MHz) (D_2O) 4.67 (HOD), 3.01 (4H, m), 1.81 (6H, s) and 1.35 p.p.m. (4H, m); m/z 172, 129, 112, 100, 87, 70, 58 and 43(100). (Found: C, 55.55; H, 9.21; N, 16.28. $C_8H_{16}N_2O_2$ requires C, 55.81; H, 9.30; N, 16.28%).

Study of Different Acylating Groups.-

Putrescine was incubated with PPL in the specified organic solvent at 25°C using the standard procedure.

(a) Ethyl formate

After a period of 48h, only diformylated putrescine could be observed. The reaction mixture was worked-up in the standard fashion, and diformylated putrescine crystallised out of ethyl formate.

Diformylated putrescine

R_f 0.81; m.p. 94-96°C; ν_{max} (KBr disc) 3280, 1630, 1540, 1390 and 1222 cm^{-1}; δ_H (90 MHz) (D_2O) 8.53 (2H, s), 5.17 (HOD), 3.75 (4H, m) and 2.05 p.p.m. (4H, m); m/z 145, 115, 99, 86, 73, 58, 46 and 30(100). (Found: C, 50.01; H, 8.31; N, 19.34. $C_6H_{12}N_2O_2$ requires C, 50.00; H, 8.33; N, 19.44%).

(b) Ethyl acetate

Details are as previously reported.

(c) Vinyl acetate

After 5h, diacylated and monoacylated putrescine were observed. Polymeric material was also visible.

(d) Ethyl propionate

The reaction was terminated after 15 days to yield 41% of monoacylated putrescine. No diacylated putrescine was observed. This product was unstable, and on attempted crystallisation, hydrolysed to yield putrescine dihydrochloride.

(e) Ethyl benzoate

After 16 days, only starting material was visible. The reaction was terminated after 30 days, when a small quantity of monoacylated putrescine was visible. Due to the difficulty in removing the ester, the product was not isolated.

Attempts to Resolve Racemic Diamines

(±)-trans-1,2-Diaminocyclohexane dihydrochloride (155).-

R_f (free base) 0.22; m.p. > 320°C (dec); ν_{max} (KBr disc) 3400, 2876, 1601, 1530, 1496 and 1034 cm^{-1}; δ_H (90 MHz) (D_2O) 4.74 (HOD), 3.47 (2H, m), 2.25-1.48 p.p.m. (8H, m); m/z 114, 97, 82, 69, 56 and 43(100). (Found: C, 38.53; H, 8.53; N, 14.89; Cℓ, 37.85. $C_6H_{16}N_2C\ell_2$ requires C, 38.50; H, 8.56; N, 14.97; Cℓ, 37.97%).

(±)-Monoacylated-1,2-diaminocyclohexane hydrochloride (156).-

R_f (free base) 0.36; m.p. 278-280°C; ν_{max} (KBr disc) 3415, 2880, 1600, 1530 and 1496 cm^{-1}; δ_H (90 MHz) (D_2O) 4.94 (HOD), 3.69 (2H, m), 2.43 (3H, s) and 2.31-1.65 p.p.m. (8H, m); m/z 119, 89, 60, 45, 40 and 18(100). When the product was recrystallised and submitted for microanalysis, the data showed that the acetyl group was no longer present, and the m.s. data was that of the diamine dihydrochloride. Thus, heating of the compound caused it to hydrolyse.

(±)-1,2-Diaminopropane dihydrochloride (157).-

R_f (free base) 0.10; m.p. 187-189°C; ν_{max} (KBr disc) 3410, 2895, 1605, 1560, 1523 and 1037 cm^{-1}; δ_H (200 MHz) (D_2O) 4.67 (HOD), 3.60 (1H, m), 3.16 (2H, m) and 1.30 p.p.m. (3H, d, J 6.7 Hz); δ_C (50 MHz) 46.0, 42.7 and 16.5 p.p.m.; m/z 112, 56, 44(100) and 30.

Monoacylated (±)-1,2-diaminopropane dihydrochloride (159).-

R_f (free base) 0.26; m.p. 191-194°C; ν_{max} (KBr disc) 3420, 3255, 2940, 1630, 1550 and 1515 cm^{-1}; δ_H (200 MHz) (D_2O) 4.63 (HOD), 3.33 (1H, m), 3.23 (2H, m), 1.86 (3H, s) and 1.13 p.p.m. (3H, d, J 6.6 Hz); δ_C (50 NHz) (D_2O, Dioxan) 175.83, 48.52, 43.09, 22.82 and 16.30 p.p.m.; m/z 117, 73, 57, 44(100), 36 and 30. (Found: C, 39.16; H, 8.64; N, 18.25; Cℓ, 23.47. $C_5H_{13}N_2OC\ell$ requires C, 39.34; H, 8.52; N, 18.36; Cℓ, 23.28%).

(±)-2-Methylcadaverine dihydrochloride (160).-

R_f (free base) 0.17; m.p. 164-166°C; ν_{max} (KBr disc) 3420, 3025, 1595 and 1455 cm^{-1}; δ_H (90 MHz) (D_2O) 4.87 (HOD), 3.23 (4H, m), 1.92 (5H, m) and 1.16 p.p.m. (3H, d, J = 7 Hz); m/z 117, 87, 70, 56, 45 and 30(100).

(±)-Monoacylated-2-methylcadaverine hydrochlorides (161) and (162).-

R_f (free base) 0.35; ν_{max} (KBr disc) 3010, 2340, 1635 and 1405 cm^{-1}; δ_H (90 MHz) (D_2O) 4.88 (HOD), 3.18 (4H, m), 2.21 (3H, s), 1.75 (5H, m) and 1.04 p.p.m. (3H, d, J 7 Hz); m/z 121, 87, 73, 58, 43 and 30(100).

R_f (amine) 0.39; ν_{max} (KBr disc) 3400, 3040, 1625 and 1405 cm^{-1}; δ_H (90 MHz) (D_2O) 5.01 (HOD), 3.43 (2H, m), 3.14 (2H, m), 2.33 (3H, s), 1.74 (5H, m) and 1.24 p.p.m. (3H, d, J 7 Hz); m/z 89. 71, 56, 43 and 30(100).

(±)-Diacylated-2-methylcadaverine (163).-

R_f 0.57; ν_{max} (KBr disc) 3320, 3200, 2960, 1665, 1385, 1265 and 1110 cm^{-1}; δ_H (90 MHz) Unresolved; m/z 200 (M^+), 141, 114, 101, 87, 72, 59, 43(100) and 30.

meso-cis-1,2-Diaminocyclohexane dihydrochloride (164).-

R_f (free base) 0.15; m.p. 280°C (dec); ν_{max} (KBr disc) 3420, 2885, 1590 and 1500 cm^{-1}; δ_H (90 MHz) (D_2O) 5.01 (HOD), 4.10 (2H, t, J 6 Hz), 2.23 (4H, m) and 2.03 p.p.m. (4H, m); m/z 114, 97, 82, 69, 56, 43(100) and 30.

Monoacylated cis-1,2-diaminocyclohexane hydrochloride (165).-

R_f (free base) 0.32; m.p. 284°C (dec); ν_{max} (KBr disc) 3440, 3020, 2970, 1595 and 1500 cm^{-1}; δ_H (90 MHz) (D_2O) 4.97 (HOD) 4.03 (2H, m), 2.20 (3H, s), 2.17 (4H, m) and 1.95 p.p.m. (4H, m); m/z 114, 97, 82, 69, 56(100), 43 and 30.

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